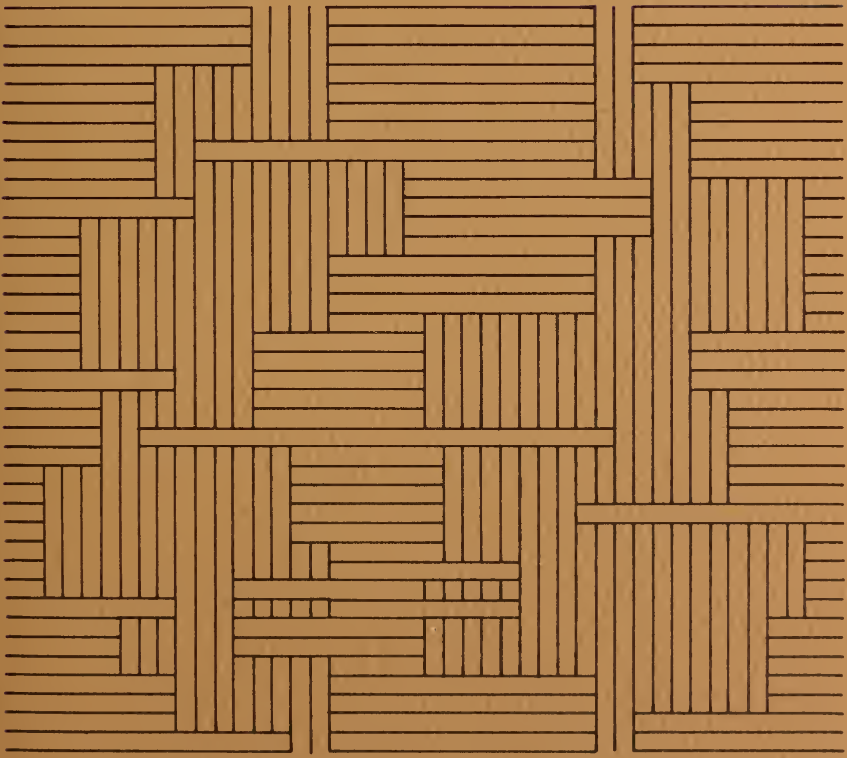
National Institute on Alcohol Abuse and Alcoholism

RESEARCH
Monograph-11

Biological Approach to Alcoholism



U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Alcohol, Drug Abuse, and Mental Health Administration

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Biological Approach to Alcoholism

Proceedings of a Conference
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Foreword

On the occasion of the inauguration of the new research building which houses the National Alcohol Research Center at the Bronx VA Medical Center, an international symposium on the "Biological Approach to Alcoholism" was held. This symposium was co-sponsored by the National Institute on Alcohol Abuse and Alcoholism (NIAAA), the Veterans Administration, and the Mt. Sinai School of Medicine. More than 300 scientists from all over the world participated by presenting their research findings, contributing to roundtable panels and by general discussions of these new research discoveries. This monograph represents a partial reporting of the proceedings of that symposium.

The National Alcohol Research Center at Mt. Sinai School of Medicine is one of nine centers funded by NIAAA. Each Center focuses on a central theme of importance to alcoholism, ranging from the neurophysiological effects of alcohol consumption to the study of factors influencing drinking patterns and trends and the prevention of alcohol problems. In addition to their primary missions of developing new knowledge by conducting original research in their chosen area within the field of alcoholism, the Centers have the further responsibility of disseminating it broadly across the scientific and lay communities. In this connection, the Centers have joined NIAAA in organizing and holding workshops and conferences on topics proximal to their specialty area. The proceedings reported in this volume represent another in the series of such collaborative activities.

The purpose of the symposium, which was held September 30-October 1, 1980, was to provide an update on the biological effects of heavy chronic consumption of alcohol. The 16 sessions, crowded into two full days, were grouped under three major headings: the Medical Complications of Alcoholism; the Metabolism of Ethanol and Acetaldehyde and Pathogenesis of Alcohol Toxicity; and Diagnosis and Treatment of Alcohol Related Diseases.

Although the size and scope of the conference was commendable in terms of its comprehensiveness, some problems became apparent as the proceedings were being prepared for publication. Because of the large number of reports and their long length in some instances, the monograph would have required two or three volumes to print the proceedings in their entirety. This was impossible because of fiscal constraints. As a consequence, a number of excellent papers could not be published at this time. Where this was necessary, we have included an abstract or summary of the paper that was omitted. In instances where the paper was subsequently submitted, in whole or in part, for publication in a professional journal, a reference has been provided so the reader can consult the full-length paper. Nevertheless, it is hoped that this monograph will help provide to the reader an update on a number of alcohol-related problems which might otherwise not be available.

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Introduction

Charles S. Lieber, M.D.
Conference Chairman

In the western world, alcohol is the most abused drug. For all the attention being directed toward heroin, cocaine, and marijuana, the favorite mood-altering drug in the United States, as it is in almost every human society, is alcohol. It is now generally recognized that alcoholism is a major (if not *the* major) social problem confronting our society. Particularly disquieting has been its steady growth. Indeed, there has been a tremendous increase of alcoholism in this country over the last three to four decades. At present, it is estimated that there are 10 to 12 million alcoholics in the United States. In our large hospital systems, such as that of the Veterans Administration, the magnitude of alcoholism and its medical complications is considered second only to that of cardiovascular disease. Actually, official statistics probably reflect underestimations because they are based on discharge diagnoses in which alcoholism is systematically underemphasized and hidden behind other terms. Thus, modern societies have not been very successful in coping with the problems associated with alcoholism as witnessed by the steady increase of alcoholism and its impact on industry (absenteeism and impaired performance) and traffic accidents: more than half of all lethal road accidents are associated with the presence of alcohol in the drivers' blood. Moreover, the medical issues related to the disease of chronic alcoholism have come to the forefront because the intrinsic toxicity of alcohol is now fully appreciated.

The prevalence of one medical problem alone, namely cirrhosis of the liver, has now reached such a magnitude that this complication of alcoholism represents, in and by itself, a major public health problem. We now recognize that 75 percent of all medical deaths attributable to alcoholism are due to cirrhosis of the liver. In the United States, cirrhosis has overtaken diabetes as the fifth leading cause of mortality; in large urban areas, it has become the third leading cause of all deaths in the active age group of 25 to 65 years. Although not all cirrhotic subjects are alcoholics, it is now generally recognized that a majority of patients with cirrhosis do admit to excessive alcohol consumption. Other tissues can also be severely affected including brain, gut, heart, endocrine systems, bone, blood, and muscle. These complications involve virtually the entire field of internal medicine and much of pathology, biochemistry, and some pharmacology. Because of its wide impact, syphilis was called "the great imitator"; this can now be said of alcoholism since its complications can mimic so many other diseases.

A question which is often raised is "in which way does an alcoholic differ from a nonalcoholic?" Inquiries have focused on psychological make-up, behavioral differences, and socioeconomic factors. More recently, however, physical differences have been delineated. Prior to the development of various disease entities, chronic ethanol exposure results in profound biochemical and morphological changes. Consequently an alcoholic does not respond normally to alcohol, other drugs, or even other toxic agents. Some of these persistent biochemical and morphologic changes are the consequences of the injurious effects of ethanol, whereas others may represent the possible adaptive responses to the profound changes in intermediary metabolism which are a direct and immediate consequence of the oxidation of ethanol itself.

The present state of the art in the biologic underpinnings of alcoholism was the subject of a conference on "Biologic Approach to Alcoholism: 1980 Update" held September 30th and October 1, 1980, at the occasion of the inauguration of the new research building of the Bronx VA Medical Center. This conference was sponsored by the Alcohol Research Center Division of the National Institute on Alcohol Abuse and Alcoholism, the Veterans Administration, and the Mount Sinai School of Medicine. The first part of the symposium consisted of a number of state-of-the-art lectures given by world experts in the field. This was followed by a number of roundtable discussions centered around major developing areas. A large poster session allowed for in-depth presentation of specific areas of research ongoing in the Center and elsewhere. Finally, recent diagnostic and therapeutic advances were the subjects of two separate sessions.

It became apparent at this conference that the significant progress made in elucidating concepts of pathogenesis and developing tools for diagnosis has opened up new avenues for prevention. As discussed in the concluding chapter, attempts at disease control at three levels of intervention can now be conceptualized: 1) biological markers of alcoholism are being developed which, when perfected, may allow for early detection of heavy drinking; 2) in these individuals at risk, improved blood tests may eventually permit detection of alcoholic liver injury and other medical disorders at reversible early stages; 3) recognition of preexisting enzyme defects or other preexisting abnormalities such as precirrhotic lesions or the detection of contributory genetic factors may permit screening for alcoholics predisposed to the development of major complications of alcoholism such as cirrhosis. A concentrated therapeutic effort on such a more manageable subgroup might arrest the disease at a potentially reversible state, prior to the social and medical disintegration of the individual. Presently, full implementation of such an approach is not yet possible; but I hope that this volume, by summarizing the advances made to date, may potentiate the progress toward such a new public health approach. The various contributions were either submitted or updated in the spring of 1981, and therefore represent the 1981 status of the various subjects of the conference.

Charles S. Lieber, M.D.

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SESSION I:
MEDICAL COMPLICATIONS OF ALCOHOLISM: AN UPDATE

Medical Complications of Alcoholism: Introduction

Thomas P. Almy

People have long viewed the injurious consequences of alcohol ingestion with skepticism—scientists being motivated by their intellectual discipline, and laymen mostly by wishful thinking. Not so long ago, for example, cirrhosis of the liver in alcoholics was classified as nutritional cirrhosis; and even today, megavitamin therapy is widely promoted.

In the past 25 years, intensive research on the intermediate metabolism of ethanol has revealed many of the mechanisms by which it interferes with the function and alters the structure of liver, pancreas, and brain; many of the myths perpetuated by the inconsistencies in earlier studies can now be dispelled. The participants in this symposium have been among the architects of this progress, and their contributions are valuable both for consolidation of present knowledge and perspectives on problems yet to be solved.

The reader will find here a summation of evidence on the pathogenesis of the alcoholic fatty liver, alcoholic hepatitis, and the early stages of cirrhosis. The contribution to the process of the acetaldehyde formed in intermediate metabolism is seen to be very significant. Observed variations in toxic effects among various classes of alcoholic beverages can now be related to the effects of impurities on aldehyde formation and degradation. The biological basis for individual and racial differences in susceptibility to the effects of alcohol is shown to be, at least in part, the result of genetic polymorphisms affecting the activity of alcohol dehydrogenase and, apparently also, aldehyde dehydrogenase.

The pathogenesis of alcoholic pancreatitis is authoritatively reviewed. Its early lesions are attributed to the precipitation of insoluble protein plugs in the major pancreatic ducts, and this in turn to excessive elaboration in the pancreatic juice of a "stone protein" with a high affinity for calcium ion.

That devastating consequence of maternal alcohol ingestion during pregnancy, the "fetal alcohol syndrome," is shown to be related at least in large part to interference by alcohol with protein synthesis in brain and other rapidly growing fetal tissues. Though several other potential influences remain incompletely explored, it is likely that this interference is due in some measure to the inhibitory effect of ethanol on placental transfer of amino acids from mother to fetus, and that of acetaldehyde on protein synthesis in the placenta.

The hepatic metabolism of alcohol is also called upon to provide clarification of its numerous and often confusing interactions with many commonly used drugs. The broad toxicological significance of these interactions is well illustrated by the dangers attending the use of a widely available analgesic, acetaminophen, by the alcoholic.

The papers which follow go far to lengthen and strengthen the chain of evidence linking alcohol to the patterns of tissue injury with which it has been clinically associated. The reader will be disappointed only if he looks for a biochemical "fix," for ways of separating the desired from the undesired effects of ethanol ingestion. He may conclude that vodka is really "the diplomat's drink" because it allows minimal accumulation of endogenous acetaldehyde. He may recommend to those with an already damaged pancreas that they moderate their intake of protein and fat. But unless the trick is turned in future research, the only reliable means for preventing the major medical complications of alcoholism will be for the fortunate, moderation, and for the unfortunate, abstinence.

Polymorphism of Human Alcohol and Aldehyde Dehydrogenase

Jean-Pierre von Wartburg

The oxidation of ethanol via acetaldehyde to acetate represents the main route of alcohol elimination in humans. It is generally agreed that the pyridin-nucleotide-dependent alcohol and aldehyde dehydrogenases in the liver represent the major pathway. The role of extrahepatic alcohol metabolism and the significance of other oxidoreductases such as the microsomal ethanol oxidizing system, catalase, and aldehyde oxidase are less well understood. Both the rate and the kinetics of the elimination of alcohol and acetaldehyde are of great importance because they determine the duration of the presence of these toxic compounds in the human body. Finally, the characteristics of alcohol and acetaldehyde metabolism are closely related to the catalytic properties of the enzymes involved in their chemical transformation.

Several research groups have observed that the activity of human liver alcohol dehydrogenase shows a considerable interindividual variation (Bosron, Li, and Vallee 1979; Li and Magnes 1975; Moser et al. 1968; Schenker et al. 1971; von Wartburg et al. 1964). This biochemical individuality can easily be explained by the occurrence of multiple molecular forms of this important enzyme, but their nature is not yet fully understood. For a large number of the multiple forms we have good evidence that they are due to the existence of isoenzymes. For additional enzyme forms enzyme polymorphisms seem to be responsible. Accordingly a genetic model has been proposed. For further forms of human alcohol dehydrogenase which have been discovered more recently the subunit composition and mode of genetic determination have not yet been elucidated.

Historical Remarks

Human liver alcohol dehydrogenase is a zinc-containing enzyme with a molecular weight of approximately 85,000 daltons and composed of two identical or nonidentical subunits. In these properties it resembles the enzymes of other species, although it may differ in the degree of heterogeneity. The human liver enzyme was first purified

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NOTE: Figures appear at end of paper.

by von Wartburg, Bethune, and Vallee (1964). This study was followed by the purification of the alcohol dehydrogenase from rhesus monkey in order to establish the properties of the enzyme from a species which might later serve as a model for in vivo studies. This led to the discovery of isoenzymes, which we reported for the first time 15 years ago (Papenberg et al. 1965). At the same time we found that this heterogeneity of alcohol dehydrogenase was not specific for the rhesus monkey; the enzymes from human and horse liver also revealed multiple molecular forms that could be demonstrated by electrophoresis and chromatography (Aebi et al. 1966; Papenberg et al. 1965; von Wartburg 1966). Our finding that human liver alcohol dehydrogenase could be separated into three bands by electrophoresis on agar gel was matched by the observation made in Vallee's laboratory which showed three fractions of the enzyme upon conventional ion exchange chromatography (Blair and Vallee 1966).

In 1965, we first published our discovery of a variant human liver alcohol dehydrogenase, which we denominated "atypical" at that time because it seemed to occur only in few individuals of the Bernese or Swiss population (von Wartburg et al. 1965). This "atypical" enzyme consisted of a mixture of isoenzymes and differed from the normal with respect to the specific activity, the pH rate profile, the substrate specificity, and the sensibility to metal binding agents.

The availability of improved methods for electrophoretic and chromatographic separation soon led to the discovery of a rapidly increasing number of multiple forms of both human and horse alcohol dehydrogenase. It was also possible to demonstrate a heterogeneity of the enzyme in other species (Moser et al. 1968). We first reported on five isoenzymes of horse liver alcohol dehydrogenase, three of which were in crystalline form (von Wartburg and Kopp 1968; von Wartburg, Kopp, and Lutstorf 1970). The characterization of the isoenzymes soon led to the proposition of a model for the subunit composition of 9 isoenzymes (Lutstorf and von Wartburg 1969), which we extended at the occasion of the eighth IUB Congress to 11 isoenzymes from horse liver and to the atypical and normal isoenzymes from human liver (von Wartburg, Bretz-Lutstorf, and Schenker 1970). Similar results were obtained independently by the groups of Theorell and Vallee, but not for human alcohol dehydrogenase (Pietruszko and Theorell 1969; Pietruszko et al. 1969).

Model for the Subunit Composition of Alcohol Dehydrogenase Isoenzymes

In analogy to our work with the horse enzyme, we proceeded to analyze the subunit composition of human liver alcohol dehydrogenase and proposed a model for six isoenzymes (Schenker et al. 1971). We suggested that these multiple forms are generated by the random combination of three different subunits (A, B, B′) to the six possible dimeric molecules (AA, AB, AB′, B′B′, B′B, and BB). The predominant isoenzyme (BB) isolated from atypical livers exhibited the catalytic features characteristic for atypical liver homogenates. Because in atypical homogenates we found an isoenzyme AB with normal characteristics in addition to the atypical isoenzyme AB, we suggested that the atypical livers came from heterozygous individuals with a mutant subunit B. Our observation was later confirmed by Pietruszko and colleagues (1972).

Subsequently our model was extended by an elegant and rather extensive study by Harris and collaborators (Smith et al. 1971, 1972, 1973*a*,*b*). It postulates the existence of three separate structural gene loci (ADH_1, ADH_2, and ADH_3) coding for three different polypeptide chains (α, β, and γ). In addition, the gene locus ADH_3 exhibits a polymorphism with two allelic genes coding for two distinct subunits γ_1 and γ_2. The atypical enzyme variant is then due to an analogous polymorphism at gene locus ADH_2 yielding the normal subunit β_1 and the atypical subunit β_2 (figure 1). We have shown that the atypical subunit differs from the normal subunit in its primary structure at least in one amino acid residue (Berger et al. 1974; Dubied et al. 1977). The subunit β_1 has an alanine residue which has been substituted for a proline in the coenzyme binding site of the atypical variant. This mutation leads to drastic changes in the functional properties. Isoenzymes containing the atypical subunit show a considerably higher specific activity and a lower pH optimum at pH 8.5 as compared to pH 10.3 for the corresponding normal isoenzymes. The genetic model predicts the existence of nine genotypes, namely three homozygous normal, three heterozygous atypical, and three homozygous atypical. Depending on the genotype, a total of 6, 10, or 15 dimeric isoenzymes can be formed by combination of the various subunits (figure 2). A diagram with all possible homodimeric and heterodimeric enzyme forms is shown in figure 3.

In addition to the multiple forms described above, a further distinct form of alcohol dehydrogenase has been identified (Bosron et al. 1977; Li and Magnes 1975; Li et al. 1977). The enzyme seems to occur in all individuals but to a variable extent. It is characterized by an anodic mobility upon starch gel electrophoresis and a relative insensitivity toward the inhibitor pyrazole. It has therefore been termed anodic form or π-enzyme. The subunit structure, zinc content, and molecular weight are similar to the other forms of the human enzyme, but the apparent Michaelis constant for ethanol is remarkably high (15-30 mM) (Bosron, Li, Dafeldecker, and Vallee 1979).

Recently, yet another form of human liver alcohol dehydrogenase was found in some individuals of a population in Indianapolis (Bosron, Li, and Vallee 1979). This form yields additional isoenzyme bands upon starch gel electrophoresis which are characterized by a more cathodic mobility and a pH optimum of 7.0. Although the analyzed sample size was rather small, the authors found an indication of a higher frequency of the cathodal form in black Americans than in white Americans. Teng et al. (1979) also observed a faster moving cathodal band in some individuals of the Chinese population but not of the Indian population living in Malaysia. Finally, it has been hypothesized that a variant observed at a low frequency in a mixed population of Brazil could be due to an allele of gene locus ADH_2 (Azevêdo et al. 1975). Altogether these observations suggest that more than one polymorphism may occur at the gene locus ADH_2 and that racial differences exist.

Both the high specific activity and the pH optimum of atypical alcohol dehydrogenase have been used as the basis for screening tests to differentiate between atypical and normal alcohol dehydrogenase in liver homogenates. The prevalence of this enzyme variant has been determined by many research groups all over the world. Our initial suggestion to use the ratio of the activities determined at the pH optima of the normal and atypical forms (von Wartburg et al. 1965) has been used in most studies aiming at a determination of the gene frequency for the atypical trait. In this way apparent bimodal distributions have been found although, theoretically, trimodal distributions would be expected. So far the attempts to separate the normal isoenzyme $\beta_1\beta_1$ from the atypical ones ($\beta_1\beta_2$ and $\beta_2\beta_2$) by chromatography have been only partially successful (Adinolfi and Hopkinson 1979; Berger et al. 1974). Both types of isoenzymes also possess the same immunological properties (Adinolfi et al. 1978). Hence, such properties cannot be used to discriminate between homozygous (ADH_22) and heterozygous (ADH_22-1) individuals. Furthermore, we have observed that some livers are difficult to classify on the basis of pH ratios and suggested determining full pH rate profiles from fresh liver material (Berger et al. 1974). Moreover, the low pH optimum of the cathodic forms represents an additional difficulty in future determinations of the frequency of the various genotypes; the results of previous studies should be considered with great caution.

Frequencies of the Polymorphic Forms

The frequency of the atypical variant has been estimated at 5 to 20 percent in Caucasian populations from Europe and the United States (Edwards and Evans 1967; Harada et al. 1978*a*; Kamaryt and

Martinek 1979; Li and Magnes 1975; Smith et al. 1973*a*; von Wartburg and Schürch 1968). In contrast, the atypical enzyme predominates in Mongolian populations and frequencies of over 85 percent have been found in Japan (Fukui and Wakasugi 1972; Harada et al. 1979; Ogata and Mizohata 1973; Stamatoyannopoulos et al. 1975; Teng et al. 1979; Tsukamoto 1973). Recently, Tsukamoto et al. (1980) attempted to determine the frequency of homozygotes and heterozygotes for the ADH_2 polymorphism on the basis of electrophoretic patterns of liver extracts purified by affinity chromatography. They observed 4.7 percent homozygous atypical ($ADH_2 2$), 14 percent homozygous normal ($ADH_2 1$), and 76.8 percent heterozygous ($ADH_2 2-1$) individuals, but 4.7 percent of the population could not be classified. However, in a similar attempt, Harada, Misawa, Agarwal, and Goedde (1980) found 40 percent of livers of Japanese to be homozygous atypical ($ADH_2 2$) and 45 percent heterozygous ($ADH_2 2-1$). This discrepancy cannot be explained at this time, especially since nothing is known about the possible occurrence of cathodic bands in Japan which might interfere with an electrophoretic procedure supposed to discriminate between homozygous and heterozygous atypical individuals. Finally, extremely low frequencies of atypical alcohol dehydrogenase also seem to occur, as observed by Teng et al. (1979) in Indians living in Malaysia and by Azevêdo et al. (1975) in a mixed population of Brazil.

Only few studies have been concerned with the frequencies of the polymorphism at gene locus ADH_3, but racial differences also seem to exist in this case. The frequency of the allele $ADH_3 1$ for the subunit γ_1 was estimated at 60 percent and 58 percent in England (Smith et al. 1972) and Germany (Harada et al. 1978*a*). In mixed populations of Indianapolis and in Bahia the frequencies are somewhat higher, with 67 percent and 86 percent, respectively (Azevêdo 1975; Bosron, Li, and Vallee 1979). A similar prevalence was found in Indian (64 percent) and an even higher value of 91 percent in Chinese (Teng et al. 1979) populations. The $ADH_3 1$ allele also seems to predominate in Japan (Tsukamoto et al. 1980). Interestingly, the average activity found in $ADH_3 2$ phenotypes is significantly higher than in $ADH_3 1$ phenotypes or heterozygotes (Bosron, Li, and Vallee 1979), although the values are still much lower in both than in atypical phenotypes.

Alcohol dehydrogenase is localized in many organs other than the liver. Most of the extrahepatic activity occurs in the top parts of the gastrointestinal tract. Some additional activity is found in the lungs, kidneys, and spleen. Only traces occur in many other tissues, including the brain (Moser et al. 1968; Tabakoff and von Wartburg 1975). The stomach mainly contains gene products of the gene locus ADH_3 (Smith et al. 1972). In a recent study two isoenzymes from human stomach have been purified and were shown to differ in their kinetic properties (Hempel and Pietruszko 1979). Hence the isoenzyme pattern depends on the organ. Furthermore, developmental variations have been observed (Adinolfi and Hopkinson 1979; Murray and Motulsky 1971; Pikkarainen and Räihä 1967; Smith et al. 1971, 1972).

In summary, a number of factors determine the remarkable variability of alcohol dehydrogenase in human liver: (1) the occurrence of genetically determined isoenzymes and enzyme polymorphisms; (2) variation in the gene expression due to developmental stages; (3) organ specificity of the isoenzyme pattern; and (4) pathological alterations in the tissue (Li 1977; von Wartburg 1971). In this context it is worthwhile to note that a high frequency of atypical alcohol dehydrogenase has been observed in alcoholics with liver disease (Kamaryt and Martinek 1979; Ugarte et al. 1970). Thus, it becomes clear that both environmental and genetic factors determine the amount and the catalytic properties of alcohol dehydrogenase. From this the question arises how the genetically controlled individual isoenzyme pattern relates to the genetic control of alcohol metabolism and of some effects of alcohol in normal subjects (Propping 1977, 1978; Vesell et al. 1971).

Isoenzyme Pattern and Alcohol Elimination

It is generally accepted that in normal humans the range of activity of liver alcohol dehydrogenase can essentially account for the range of alcohol elimination rates observed in vivo. For theoretical reasons both the alcohol dehydrogenase and the rate of reoxidation of the reduced coenzyme NADH limit the rate of alcohol oxidation (Higgins 1979; Plapp 1975). Since the activity of alcohol dehydrogenase is considerably elevated in atypical individuals or phenotypes with γ_2, one might expect that such individuals could eliminate alcohol at a faster rate than normals. However, the few atypical individuals studied were found to have only a slightly higher rate than average during the steady state of alcohol metabolism (Edwards and Evans 1967; von Wartburg and Schürch 1968). Such a slight elevation of the rate of acetaldehyde production might lead to increased levels of acetaldehyde in liver and blood, especially in individuals with relatively low activities of aldehyde dehydrogenase (see below).

The Michaelis constants (K_m) for ethanol as substrate for the various forms of human liver alcohol dehydrogenase vary considerably. The main isoenzyme $\beta_1\beta_1$ isolated from normal liver homogenates reveals nonlinear kinetics with a lower K_m of 0.5 mM and a higher K_m of approximately 8 mM, and equal contributions to the maximal velocity (Dubied et al. 1977). Hence, the higher value is similar to the one observed for the microsomal ethanol oxidizing system which has been found to be 8 to 10 mM (Lieber and DeCarli 1970). Finally, an even higher value of 15 to 30 mM has been reported for the π-enzyme (Bosron et al. 1977). From these findings the question arises as to what extent the variable affinities of the multiple enzyme forms also affect alcohol elimination in vivo.

According to the classical work of Widmark and of many other investigators, blood alcohol elimination curves approximate linearity and remain independent from the ethanol concentration until very low concentrations are reached. However, this concept of linearity has been challenged by a number of researchers who observed a concentration dependence of blood alcohol clearance. Lundquist and Wolthers (1958) were the first authors who suggested that the pharmacokinetics of ethanol follow Michaelis-Menten kinetics. More recently Wagner and collaborators proposed a new model based on the Michaelis-Menten equation (for two recent reviews see Sturtevant and Sturtevant [1979] and Wilkinson [1980]). These models, however, assume only one Michaelis-Menten constant. Furthermore, they do not account for the possibility that alcohol could be removed at an enhanced rate during the phase of absorption from the gastrointestinal tract. The relatively high alcohol concentrations in the portal vein should lead to increasing saturation of the high K_m forms of alcohol dehydrogenase, so that a considerable amount of alcohol is removed by the first pass elimination during absorption. Alcohol eliminated by such a mechanism will not appear in the peripheral blood, although it has been absorbed in the gastrointestinal tract. Hence, an apparent incomplete absorption will result from this phenomenon and the area under the peripheral blood alcohol curve will be diminished in spite of a complete absorption.

In order to evaluate the extent to which the variable kinetic properties of the isoenzyme patterns could theoretically modify the blood elimination curves, we carried out some computer simulations. In these computer-simulated blood alcohol curves, both the complex kinetic parameters and the first pass elimination were accounted for. The calculations were based on the following assumptions: Total alcohol dose 50 or 100 g; total distribution volume 50 l; absorption of alcohol from the gastrointestinal tract occurs exponentially, half of the quantity present at a given time being removed in 10 min; blood flow through portal vein 1 l/min; total blood elimination rate 0.2 mg/ml·h at a blood alcohol concentration of 1 mg/ml; blood alcohol elimination rates (ER) following Michaelis-Menten kinetics according to the equation $ER = Vmax \cdot c(t)/K_m + c(t)$; c(t) being the blood alcohol concentration at a given time t; the blood alcohol concentration in the portal vein was calculated as the sum of the alcohol concentration resulting from absorption and from circulation in the peripheral blood; Michaelis constants for ADH=0.5 and 8 mM, for π—ADH=20 mM, and for MEOS=8 mM; the percentage contribution of the single enzyme forms to the total elimination rate was varied. The peripheral blood alcohol concentrations were plotted versus time on an X-Y-plotter.

As can be seen in figure 4, the resulting blood alcohol curves are clearly nonlinear over the whole range of blood alcohol levels and the rate of alcohol elimination is concentration dependent. Nevertheless, for practical purposes the decrease of blood alcohol during 1 given hour could be considered to approximate linearity. An increase of the alcohol dose from 50 to 100 g leads to higher blood alcohol levels at which the elimination rate is increased. For the blood alcohol curves shown in figure 5, varying total elimination rates and enzyme patterns have been assumed. It is obvious that the area under the curve decreases with increasing total elimination rates. The increasing first pass elimination becomes evident from the decreasing and earlier peak concentrations. For curves 2 and 3, the total elimination rate was kept constant (0.2 mg/ml·h), but the percentage of the π-enzyme participation with its high K_m was increased from 10 percent (curve 2) to 40 percent (curve 3). The curves show relatively little difference at blood alcohol levels above 0.5 mg/ml and a slightly slower fall at lower blood alcohol concentrations, due to less saturation of the ADH isoenzyme mixture. As judged from these curves, it seems questionable whether such differences could be found by least square fitting of actual experimental data obtained from drinking experiments. The curves shown in figure 6 were calculated with an elimination rate of 0.2 mg/ml·h and a participation of 20 percent π-ADH. It can clearly be seen that the first pass elimination becomes considerable when a slower rate of absorption is assumed. This indicates that the consumption of food, for instance, by slowing down the rate of absorption allows for better saturation of the high K_m forms for a longer period of time, thus efficiently removing alcohol before it reaches the peripheral blood.

Isoenzymes of Aldehyde Dehydrogenase

In comparison with human liver alcohol dehydrogenase, surprisingly little is known about the human aldehyde dehydrogenase and its genetic regulation. The enzymes from other species such as horse, ovis, or rat have been investigated more extensively and from various points of view; several reviews deal with the metabolism of acetaldehyde and its enzymes (Li 1977; Lindros 1978; Weiner 1979*a,b*). Acetaldehyde as a toxic intermediary metabolite of alcohol metabolism has been implicated in a great number of effects of acute and chronic alcohol intoxication (Lieber 1977; von Wartburg 1980*a*). Hence, the levels of acetaldehyde in organs and in the blood are of great importance. During alcohol metabolism the steady state concentration of acetaldehyde will reflect its rates of formation and of removal. Elevated acetaldehyde levels in the blood would therefore be expected in individuals with especially high activity of alcohol dehydrogenase (and/or MEOS) or low activity of aldehyde dehydrogenase or a combination of both.

Several research groups have found increased concentrations of acetaldehyde in the blood of Orientals who suffer from alcohol intolerance (Asakura 1980; Jiri 1974; Kinoshita 1974; Mizoi and Ohga 1963; Mizoi et al. 1978; von Wartburg 1980*b*). Since this high frequency of hypersensitivity to alcohol observed in Oriental populations coincides with the high frequency of atypical alcohol dehydrogenase, the hypothesis has been advanced that these individual and racial differences in alcohol sensitivity are partly determined by the polymorphism of alcohol dehydrogenase (Stamatoyannopoulos et al. 1975; von Wartburg 1976, 1977; von Wartburg et al. 1975). However, in Japanese individuals with noticeable flushing, Inoue et al. (1980) observed a negative correlation between the elevated acetaldehyde levels and the activity of aldehyde dehydrogenase in the erythrocytes. In view of the high frequency of atypical alcohol dehydrogenase in Japan, these individuals may accumulate acetaldehyde by both a faster formation and a slower oxidation.

It would, therefore, be of interest to know whether such a correlation also occurs in populations with low frequencies of high activity alcohol dehydrogenase phenotypes. Although the relation between the aldehyde dehydrogenase in erythrocytes and the enzymes in liver is unclear, these observations may emphasize the role of the erythrocytes as a potential metabolic compartment for acetaldehyde removal. Activities between 5 and 50 μmoles/min·ml blood and two K_m in the micromolar and millimolar range have been reported (Inoue et al. 1978, 1979; Pietruszko and Vallari 1978; von Wartburg 1980*a*). This activity appears little when compared to the one at the main localizations of this enzyme, namely in liver and kidney. Nevertheless, an average removal of only 5 μmoles/min·ml corresponds to a total capacity of 25 μmoles/min for 5 l of blood. At an acetaldehyde concentration of 10 μmoles/l, this represents half of the amount of acetaldehyde present in blood. This is a considerable rate of disappearance which might be of physiological relevance if it also occurs in vivo, especially since the amount of acetaldehyde transported to organs such as the brain or the adrenal glands would also depend on the amount removed during the time required for transport in the blood. Finally, removal of acetaldehyde during sampling may seriously interfere with the determination of acetaldehyde in human blood if no adequate precautions are taken (von Wartburg and Ris 1979).

Aldehyde dehydrogenase from human liver was first isolated by Kraemer and Deitrich (1968). Evidence for the existence of isoenzymes was then obtained by Blair and Bodley (1969), who observed a major and a minor peak upon chromatography on DEAE-cellulose. More recently, two isoenzymes were purified to homogeneity from human liver extracts by the use of affinity chromatography (Greenfield and Pietruszko 1977). From these investigations and from a study of the subcellular distribution (Koivula 1975), it appears that at least three distinct forms of human aldehyde dehydrogenases can be distinguished: (1) a cytosolic isoenzyme with slow electrophoretic mobility, high K_m for acetaldehyde, and high sensitivity to the inhibitor disulfiram; (2) a mitochondrial isoenzyme with faster electrophoretic mobility, low K_m to acetaldehyde, and less sensitivity to disulfiram; and (3) a minor form localized in the microsomal fraction. In addition, a chromatographic heterogeneity of the mitochondrial enzyme was observed. Similar to the enzymes from other species, the human isoenzymes seem to have a tetrameric structure with molecular weights around 240'000-250'000. It is generally agreed that the mitochondrial enzyme with low K_m for acetaldehyde is responsible for most of the oxidation of acetaldehyde produced in the cytosol by alcohol dehydrogenase (Lindros 1978; Weiner 1979*a*).

A much higher degree of complexity for the multiple molecular forms of human aldehyde dehydrogenase and the existence of an enzyme polymorphism in Japanese individuals has recently been proposed by Goedde and his collaborators. In a first study using starch gel electrophoresis, 68 liver and kidney specimens and 5 other tissues were analyzed (Harada et al. 1978*a*). The authors found a total of nine enzyme bands and classified them into three isoenzyme sets. From this, five different phenotypes were derived and preliminary gene frequencies were calculated for the proposed three gene loci. In a further study using isoelectric focusing, the multiple bands were interpreted as to represent up to four sets of aldehyde dehydrogenase (Goedde et al. 1979*a*). Using starch gel electrophoresis and isoelectric focusing, the same authors have identified four major isoenzymes from human tissues and attempted partial purification and characterization (Harada, Agarwal, and Goedde 1980). Two of these isoenzymes seem to correspond to the ones previously characterized by Greenfield and Pietruszko (1977). In view of the varying number of isoenzymes reported, the possible artifactual formation of bands, and the lack of knowledge on the subunit composition of the isoenzymes, it seems premature to speculate on the genetic regulation of human aldehyde dehydrogenase at this time.

Polymorphism of Aldehyde Dehydrogenase

A further observation by Goedde and his group concerning a possible polymorphism of the enzyme is of great interest. The authors analyzed 40 autopsy livers from Japanese subjects (Harada et al. 1979; Harada, Misawa, Agarwal, and Goedde 1980) and found that 48 percent of the individuals revealed two major isoenzyme bands ("usual" phenotype), whereas 52 percent of the samples had an "unusual" phenotype with only the slower migrating isoenzyme. In their similar study of 68 specimens from Germany (Harada et al. 1978*b*), no such polymorphism was observed. The "unusual" phenotype showed a lower mean activity than the usual, exhibited a high K_m for acetaldehyde, and disulfiram was a strong inhibitor of the activity. It is not yet clear how these findings relate to the bimodal distribution of aldehyde dehydrogenase activity in erythrocytes observed by Inoue et al. (1980), especially because Harada et al. (1978*b*) observed only one electrophoretic band in erythrocytes as opposed to the two forms shown by Pietruszko and Vallari (1979). They are also at variance with previous work of Stamatoyannopoulos et al. (1978) who found no such polymorphism in their Japanese liver samples. Nevertheless, these observations are of great importance because they offer an alternative explanation for the ethnic variations in alcohol sensitivity (Goedde et al. 1979*b*). Further studies will be necessary to see whether individuals with the "unusual" phenotype of aldehyde dehydrogenase but normal alcohol dehydrogenase exhibit the flushing syndrome. Such individuals will be rare in Oriental populations because of the high frequency of atypical alcohol dehydrogenase, and in Caucasians because of the low frequency of "unusual" aldehyde dehydrogenase.

These observations on the multiple molecular forms of human alcohol and aldehyde dehydrogenase and the polymorphisms offer a biological basis for a better understanding of the individual and racial differences in sensitivity to alcohol. It appears that elevated levels of acetaldehyde represent a major link in the processes leading to facial flushing, tachycardia, headache, muscle weakness, nausea, and sleepiness after the ingestion of relatively small amounts of alcohol. Elevated levels of the toxic acetaldehyde may also play an important role in a faster and more pronounced development of disorders associated with chronic alcohol intake. Aversive symptoms may not only influence individual drinking patterns but also codetermine drinking habits in whole populations. It would therefore be of interest to search for correlations between such biologic data and epidemiologic, demographic, and cultural data by comparing Caucasian and Oriental populations in cross-cultural studies.

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Figure 1. **Multiple Molecular Forms of Human Liver Alcohol Dehydrogenase: Genetic Model and Subunit Composition of the Isoenzymes**

gene locus	subunit	multiple molecular forms
		isoenzymes
1	○ α	○○ ◍◍ □□
2	◍ β	
3	□ γ	○◍ ○□ ◍□
2 allele 1	◍ β_1 "normal"	polymorphism → additional "atypical" isoenzymes
allele 2	⊗ β_2 "atypical"	○⊗ ◍⊗ □⊗ ⊗⊗

Figure 2. **Isoenzymes of Human Liver Alcohol Dehydrogenase in Various Genotypes**

ADH$_1$		ADH$_3$		
		1	1 − 2	2
ADH$_2$	1	6	10	6
	1−2	10	15	10
	2	6	10	6

ADH$_1$, ADH$_2$, and ADH$_3$ represent the 3 gene loci for different subunits. Types 1 and 2 are homozygous, types 1 - 2, heterozygous. The figures indicate the number of isoenzymes of alcohol dehydrogenase.

Figure 3. Schematic Representation of the Electrophoretic Pattern and Subunit Composition of Human Liver Alcohol Dehydrogenase Isoenzymes

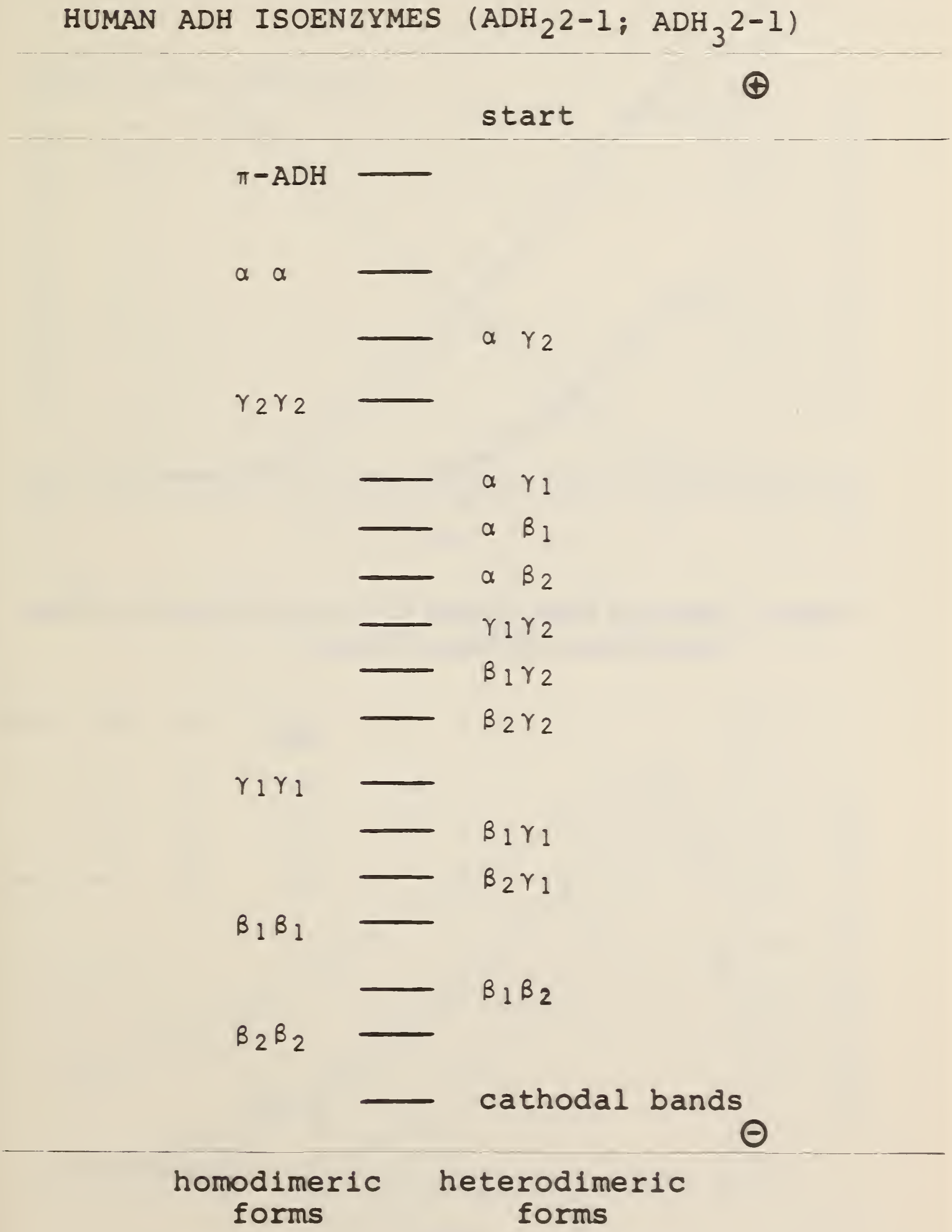


Figure 4. Simulated Blood Alcohol Curves After Ingestion of 50 g (Curve 1) or 100 g (Curve 2) of Alcohol

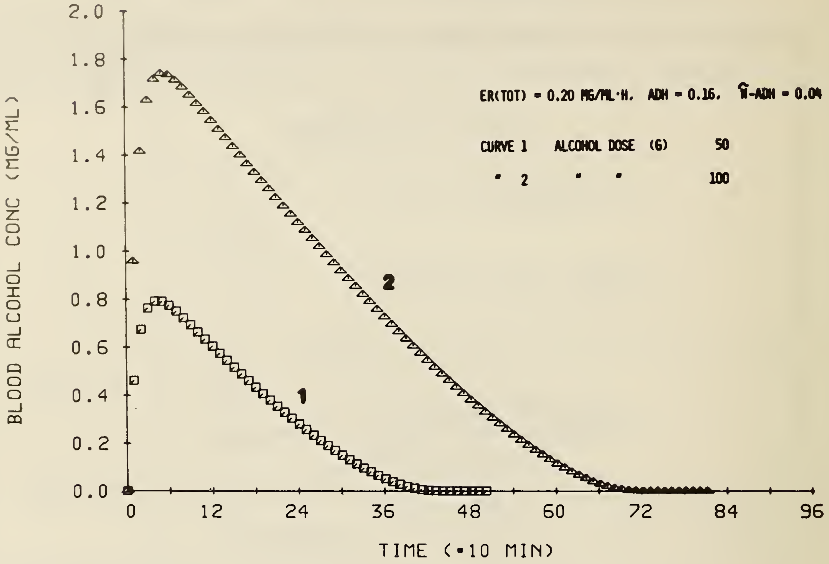


Figure 5. Simulated Blood Alcohol Curves for Varying Total Elimination Rates and Enzyme Patterns

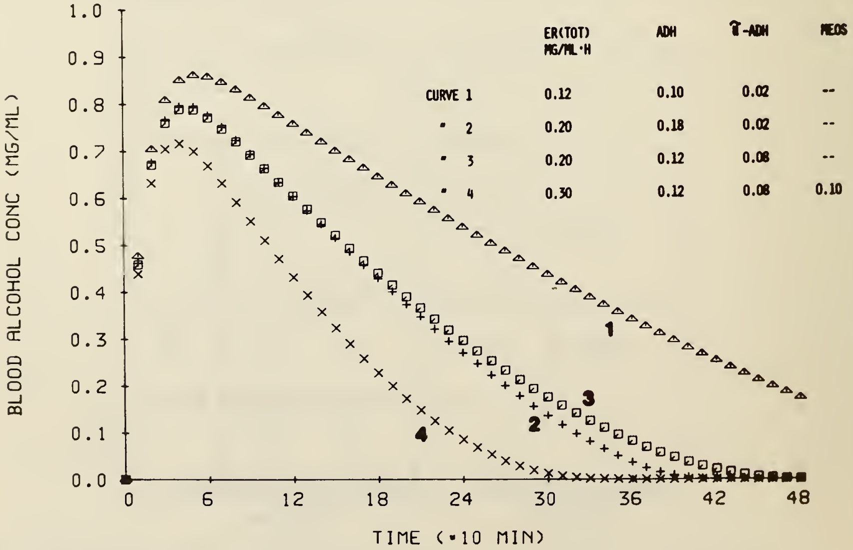
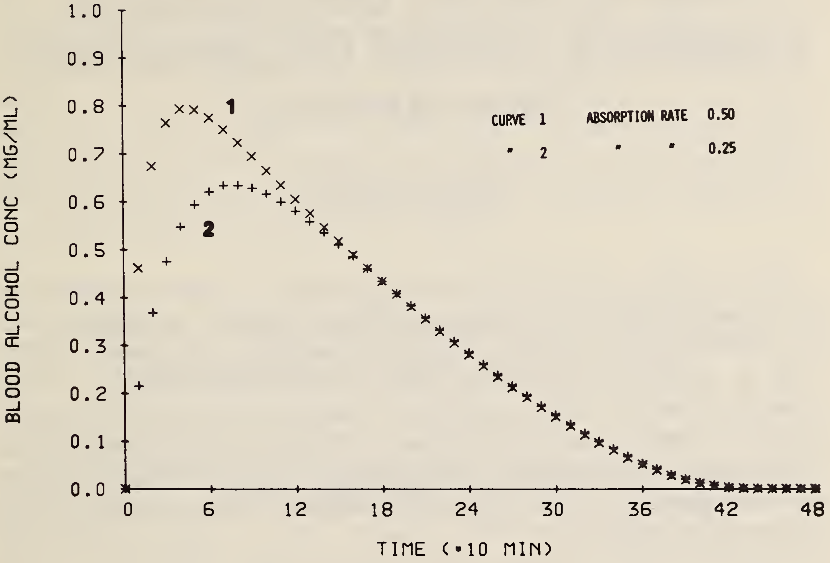


Figure 6. **Simulated Blood Alcohol Curves for Varying Absorption Rates**



Occurrence of Alcohol Dehydrogenase in Tissues Other Than Liver and Developmental Changes in Alcohol Dehydrogenase Gene Activity

Moyra Smith

Most studies on the biochemical properties of human alcohol dehydrogenase have been carried out using adult liver as the source of the enzyme (Bosron and Li 1979; von Wartburg and Schürch 1968). ADH is, however, readily detectable in other human tissues such as stomach, intestine, lung, and kidney. Analyses of the ADH isozymes present in these tissues, and analyses of the developmental changes in ADH expression during fetal and postnatal life, have contributed to our understanding of the origin of the complex ADH isozyme patterns observed in adult liver.

ADH in Fetal Liver: Expression of the ADH_1 Gene Locus

During the first trimester of fetal life, a single ADH isozyme occurs in fetal liver. In the classification of Smith et al. (1971, 1972), this isozyme was attributed to the expression of the ADH1 gene locus. During the second and third trimesters, two additional isozymes occur which differ from the isozyme observed in early fetal life, in terms of electrophoretic mobility. These isozymes are due to activity of a separate ADH locus, the ADH2 gene locus (Smith et al. 1971, 1972). The ADH1 gene product has been found to constitute the major ADH isozyme in certain primary hepatomas (Smith 1972). The ADH1 gene product differs from the products of the other ADH loci in several of its properties: It is relatively less stable and it is most active with allyl alcohol and with chloral hydrate (Smith 1972; Smith et al. 1973).

ADH in Lung: Expression of the ADH_2 Gene Locus

In lung tissue from fetuses, infants, children, and adults, ADH occurs as a single isozyme. Lung ADH has been shown to be the product of the ADH2 locus in the classification of Smith et al. (1971, 1972). The ADH2 gene is polymorphic. In approximately 10 percent of Caucasians, a variant form of the ADH2 gene product occurs, which differs from the usual ADH2 gene product in stability, pH optima, and sensitivity to inhibitors (von Wartburg and Schurch 1968). This variant form of ADH1, termed atypical, also differs in its electrophoretic mobility (Smith et al. 1973). The so-called atypical ADH1 gene has been found to represent the most common ADH1 gene allele in certain Oriental populations, particularly the Japanese (Harada et al. 1980; Stamatoyannopoulos et al. 1975).

ADH in Stomach, Intestine, and Kidney: Expression of ADH_3 Gene Locus

From early fetal life onward, the ADH activity in stomach and intestine is primarily due to expression of a separate gene locus termed ADH3 (Smith et al. 1971, 1972). This gene locus is also expressed in fetal kidney while it is only weakly expressed in adult kidney. The ADH3 gene products differ from the other forms of ADH in substrate specificity (ADH3 products are more active with amyl or butanol than with ethanol), and in sensitivity to inhibitors (ADH3 gene products are more susceptible to inhibition with isobutyramide than other forms of ADH). The ADH3 gene is highly polymorphic. On the basis of electrophoretic mobility, three common phenotypes can be observed. These phenotypes are due to the occurrence of two common alleles, ADH3 1 and ADH3 2. Differences in the frequency of these two alleles exist in different populations (Smith et al. 1972; Teng et al. 1979). Differences in the activity of the ADH3 gene products have been observed and these differences may be due to additional allelic variation at the ADH3 gene locus (Smith 1972).

ADH Isozymes in Liver in Children and Adults

In liver tissue in children and adults all of the ADH loci described above are expressed. A large number of isozymes exist because an ADH polypeptide chain may associate with (a) an identical polypeptide derived from the same gene locus, (b) an unlike polypeptide derived from an alternative allele at the same gene locus, or (c) a polypeptide derived from another gene locus. An additional form of ADH has been identified in biopsy samples of liver. This form, which is probably due to expression of a fourth ADH locus, is much less stable than other forms of ADH and also differs from them in electrophoretic mobility, Km for ethanol, and susceptibility to pyrazole inhibition (Li and Magnes 1975). This so-called Pi ADH does not form heterodimers with the other ADH gene products.

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Alcohol and Drug Interactions: Basic Concepts

Sten Orrenius

Metabolic interactions between ethanol and various commonly used drugs have long been recognized and also found to be of clinical significance. Since the liver is the main site for the biotransformation of both alcohol and drugs, mechanisms responsible for their metabolic interactions have been studied primarily in hepatic tissue. As a result of this research it has now become apparent that the hepatic metabolism of ethanol and various drugs may share certain enzymes and coenzymes, and that effects on such common components can explain many of the metabolic interactions observed. It is the purpose of this presentation to discuss some biochemical mechanisms responsible for metabolic interactions between ethanol and drugs and to comment on the possible physiological and toxicological implications of such interactions.

Hepatic Metabolism of Drugs and Ethanol

Metabolism of Drugs

Some of the more important reactions involved in hepatic drug biotransformation are illustrated schematically in figure 1. Most often, lipid-soluble drugs undergo both phase I and phase II biotransformation reactions before being excreted as polar metabolites (Parke 1968). Phase I reactions include oxidation, reduction, and hydrolysis, whereas phase II reactions are of synthetic nature and involve formation of conjugates with endogenous acid or carbohydrate. Both phase I and phase II reactions often depend on the availability of cytosolic cofactors, such as reduced nicotinamide adenine nucleotides and glutathione, activated forms of sulfate and glucuronic acid, and so forth. Phase II reactions, in particular, appear sometimes to be limited by the access to cofactors. This is true for both sulfate and glucuronic acid conjugations, as well as for glutathione conjugation under extreme conditions such as overdoses of certain drugs (Aitio 1979; Jollow et al. 1977).

NOTE: Figures and tables appear at end of paper.

The cytochrome P-450-linked monooxygenase has attracted considerable interest during recent years, and more research has been devoted to this system than to any other enzyme involved in drug metabolism (Sato and Omura 1978; White and Coon 1980). This is partly because it has become increasingly clear that many cytochrome P-450-catalyzed reactions result in formation of reactive metabolites which may cause acute or chronic toxicity, including tumorigenesis.

It is now apparent that hepatic cytochrome P-450 consists of several isozymes with different but overlapping substrate specificity, which respond differently to various inhibitors and inducers (Estabrook and Lindenlaub 1979). The existence of multiple subspecies with different substrate specificity and inducibility does in fact appear to be a common feature for several drug-metabolizing enzymes in addition to cytochrome P-450, including glucuronyl transferase and glutathione S-transferase (Aitio 1979). Hepatic sulfotransferase, which has not yet been shown to be inducible, also appears to exist in more than one form (Sekura and Jakoby 1979), which is also true for epoxide hydrolase. Like glutathione S-transferase, the latter enzyme appears to be present in both the endoplasmic reticular and cytosolic compartments (Bridges and Chasseaud 1979).

A requirement for both NADPH and molecular oxygen is characteristic of cytochrome P-450-linked monooxygenase reactions. However, the affinity of the monooxygenase is high for both NADPH and O_2, and there is no evidence that cytochrome P-450-catalyzed reactions are limited by lack of NADPH under physiological conditions. Moreover, the capacity of the hepatocyte to regenerate NADPH is extensive with its access to at least three major mechanisms for this purpose, i.e., the α-ketoglutarate—isocitrate shuttle, the pentose phosphate pathway, and malic enzyme (Sies et al. 1979).

For quite some time NADH has been known to have a synergistic effect on several cytochrome P-450-linked drug monooxygenation reactions in liver microsomes (Conney et al. 1957; Cohen and Estabrook 1971). Although the mechanism of NADH synergism has not yet been conclusively established, the available evidence suggests that it involves facilitated reduction of the cytochrome P-450-oxy complex by donation of the second electron via cytochrome b_5 (Hildebrandt and Estabrook 1971). As discussed below, there is recent evidence that this may act to stabilize the cytochrome P-450-oxy complex and diminish the release of O_2^- during the reaction cycle (Ingelman-Sundberg and Glaumann 1980). However, the evidence supporting this function of cytochrome b_5 in cytochrome P-450-catalyzed monooxygenase reactions is derived from experiments with microsomes or the purified, reconstituted enzyme system, and the significance of this mechanism for the in vivo situation still remains to be demonstrated.

Metabolism of Ethanol

Ethanol is rapidly degraded in the liver and there is general agreement that the major pathway for its metabolism involves alcohol dehydrogenase, an enzyme present in the soluble part of the cytoplasm that catalyzes the oxidation of ethanol to acetaldehyde in the presence of NAD^+ (figure 2). The acetaldehyde formed is further oxidized to acetate by aldehyde dehydrogenase present primarily in the mitochondrial compartment. Under normal conditions, the further metabolism of acetaldehyde is rapid and the first step, i.e., the oxidation of ethanol to acetaldehyde, is rate limiting for overall ethanol metabolism. Moreover, the affinity of ethanol for binding to alcohol dehydrogenase is so high that the enzyme is saturated even at low ethanol concentrations, a phenomenon which is reflected by the almost complete linearity of ethanol disappearance usually observed in vivo.

The dissociation of the enzyme—NADH complex was early shown to be the rate-limiting step in alcohol dehydrogenase-catalyzed ethanol oxidation (Theorell and Chance 1951). In the intact liver cell this dissociation rate is regulated by the cytosolic NADH level, and indeed the reoxidation of NADH has been found to be rate determining for overall ethanol metabolism (Grunnet et al. 1973; Tygstrup et al. 1974; Videla and Israel 1970). Reoxidation of cytosolic NADH is mediated via the mitochondrial respiratory chain after shuttling of reducing equivalents into the mitochondrial compartment. Due to the limited capacity of the various hydrogen shuttle systems, the redox level of the cytosolic $NADH/NAD^+$ couple becomes shifted toward the reduced state during ethanol metabolism (Forsander 1970), and this shift toward higher levels of cytosolic NADH has in fact been considered to be responsible for many of the adverse effects on the liver caused by prolonged ingestion of alcohol.

In addition to alcohol dehydrogenase, there exist at least two other systems that can contribute to hepatic ethanol oxidation (figure 2). In the presence of hydrogen peroxide, catalase has long been known to catalyze the oxidation of both methanol and ethanol to the corresponding aldehydes (Keilin and Hartree 1945). Since H_2O_2 is a normal cellular metabolite formed in a variety of metabolic reactions, including the autooxidation of cytochrome P-450, it appears conceivable that the peroxidatic activity of catalase may contribute to alcohol oxidation in the liver cell. Even though the extent of this contribution may be minimal under normal conditions, the contribution of catalase to hepatic alcohol oxidation could become substantial under conditions of enhanced intracellular H_2O_2 generation (see below).

The existence of a microsomal ethanol oxidizing system (MEOS) was first suggested by the observation that ethanol feeding induces a proliferation of hepatocyte smooth endoplasmic reticulum and enhanced rate of ethanol oxidation in the isolated liver microsomal fraction (Iseri et al. 1964; Lieber and DeCarli 1968). The fact that this fraction is normally contaminated with peroxisomes, and hence with catalase, and generates H_2O_2 during aerobic incubation with NADPH (Gillette et al. 1957) led several investigators to postulate that NADPH-supported microsomal ethanol oxidation is merely a function of the peroxidatic activity of contaminating catalase (Thurman et al. 1972). However, during the last few years it has become increasingly clear that the catalase pathway cannot account for all of the NADPH-linked ethanol-oxidizing activity of isolated liver microsomes, and that there also exists a catalase-independent mechanism which involves cytochrome P-450 (figure 2) (Lieber 1975). Evidence for the existence of the latter mechanism is derived primarily from experiments with the purified and reconstituted cytochrome P-450 system, which suggest that there exists a special form of liver microsomal cytochrome P-450 that is active in ethanol oxidation (Ohnishi and Lieber 1977). The possibility that this reaction is mediated by hydroxyl radicals generated by the cytochrome P-450 system has recently been considered by Cederbaum and his colleagues (1978). However, further experimental evidence is required to clarify the exact nature of cytochrome P-450-mediated alcohol oxidation.

Metabolic Interactions Between Ethanol and Drugs

From the discussion above it is obvious that there exist several possible mechanisms for metabolic interactions between alcohol and drugs. Some of these mechanisms are listed in table 1. As shown in this illustration, each of the three major systems known to be capable of ethanol oxidation may be affected by the presence of drugs. Conversely, ethanol may influence several of the major drug biotransformation reactions. In both cases, the metabolic interaction may involve either a specific cofactor required for the reaction or a direct effect on the responsible catalyst and may lead to stimulation or to inhibition of biotransformation. In this presentation, I shall first briefly consider possible effects of drugs on ethanol oxidation and then summarize some of the mechanisms by which ethanol may influence drug metabolism. Some physiological and toxicological implications of these metabolic interactions are discussed in a concluding section.

Effects of Drugs on Ethanol Oxidation

Available evidence indicates that alcohol dehydrogenase is the catalyst primarily responsible for the hepatic oxidation of ethanol to acetaldehyde which, in turn, is rapidly further oxidized to acetate by aldehyde dehydrogenase. Under normal conditions there appears to be no accumulation of acetaldehyde during ethanol metabolism in the liver, and the first step in the reaction sequence, i.e., the conversion of ethanol to acetaldehyde, seems to be rate determining in the overall degradation of ethanol.

The rate of ethanol metabolism by alcohol and aldehyde dehydrogenases may be affected by competing endogenous substrates as well as by various drugs. Neither enzyme is specific for ethanol or acetaldehyde, but also catalyzes the oxidation of many other endogenous as well as exogenous compounds including several alcohols and aldehydes, steroids, and ω-hydroxy fatty acids (Björkhem 1972). Several drugs act as potent inhibitors of alcohol or aldehyde dehydrogenases. The pyrazole derivatives (Theorell et al. 1969) and disulfiram are the best known of these drugs, and disulfiram in particular has long been used in the treatment of alcoholism (Haley 1979). Many experimental as well as clinical studies also show marked effects on ethanol metabolism in the presence of drugs which can act as selective inhibitors of alcohol or aldehyde dehydrogenases (Blomstrand and Theorell 1970; Haley 1979).

During alcohol dehydrogenase-catalyzed metabolism of ethanol in the liver, the redox level of the $NADH/NAD^+$ couple is shifted considerably toward the reduced state as hydrogen is transferred from ethanol to NAD^+ in the course of the reaction (Forsander 1970). The dissociation of the alcohol dehydrogenase—NADH complex is rate limiting for the activity of the isolated enzyme and is very sensitive to inhibition by increased NADH levels (Theorell and Chance 1951). In accordance with this, the reoxidation of cytosolic NADH seems to be rate determining for ethanol metabolism in liver slices and isolated hepatocytes as well as in vivo, and hence any reaction leading to enhanced oxidation of cytosolic NADH, or direct oxidation of the alcohol dehydrogenase—NADH complex, should also stimulate the rate of ethanol oxidation under these conditions (Grunnet et al. 1973; Tygstrup et al. 1974; Videla and Israel 1970).

In studies with liver microsomes, NADH has been shown to affect cytochrome P-450-mediated monooxygenase reactions, stimulating drug metabolism in synergism with NADPH (Cohen and Estabrook 1971; Conney et al. 1957). While several different mechanisms have been proposed to explain this phenomenon, the most plausible explanation for the NADH effect involves the donation of electrons via the NADH-linked flavoprotein and cytochrome b_5 to the reduced, oxygenated cytochrome P-450-substrate complex (figure 3). Although NADPH is obviously capable of donating both electrons required in the cytochrome P-450-mediated monooxygenase reaction, there is recent evidence that the cytochrome P-450-oxy complex is stabilized and the overall reaction stimulated in the presence of reduced cytochrome b_5 (Ingelman-Sundberg and Glaumann 1980).

In accordance with the above hypothesis, drugs undergoing monooxygenation should stimulate the oxidation of ethanol by alcohol dehydrogenase, provided that electrons deriving from NADH are utilized in the monooxygenase reaction. This appears to be the case under certain conditions. Thus, the adrenergic β-receptor blocking drugs alprenolol and propranolol as well as hexobarbital were shown to stimulate ethanol oxidation in isolated rat hepatocytes (Grundin 1975). The stimulatory effect was abolished by 4-methylpyrazole and was associated with a decrease in the cellular lactate/pyruvate ratio, indicating that it was probably related to an enhanced rate of reoxidation of cytosolic NADH in presence of the drugs tested. It should be noted, however, that drug stimulation of ethanol oxidation was observed only at low ethanol concentrations, indicating that only then was ethanol oxidation limited by the rate of reoxidation of cytosolic NADH (Grundin 1975).

Chronic ethanol consumption has long been known to result in an enhanced metabolism of both ethanol and certain drugs (Lieber 1975). On the other hand, no firm evidence indicates that this stimulation of ethanol oxidation is due to either induction of alcohol dehydrogenase or facilitated reoxidation of cytosolic NADH during ethanol metabolism. Thus, it was close at hand to interpret the observation by Lieber and his colleagues some 15 years ago that ethanol feeding causes a proliferation of hepatic smooth endoplasmic reticulum and enhanced NADPH-linked ethanol oxidation by the isolated liver microsomal fraction as evidence for the existence of a substrate-inducible microsomal ethanol-oxidizing system (Lieber and DeCarli 1968). The inability to inhibit hepatic ethanol oxidation completely by potent inhibitors of alcohol dehydrogenase, such as the pyrazole derivatives, provided further evidence for the existence of other pathways of hepatic ethanol oxidation in addition to alcohol dehydrogenase (Grunnet et al. 1973; Pappenberg et al. 1970). After several years of intense debate over the nature of this residual activity, it now appears safe to conclude that it represents the sum of ethanol oxidation activities catalyzed by cytochrome P-450 and the peroxidatic function of catalase (Cedarbaum et al. 1978).

Detailed studies of the induction of the hepatic microsomal ethanol-oxidizing system by ethanol feeding have revealed that it is associated with enhanced levels of cytochrome P-450 and NADPH-cytochrome P-450 reductase and with an increase in cytochrome P-450-linked monooxygenation of several drug substrates (Lieber 1975). The latter phenomenon is probably the reason for the well-known increase in drug metabolism observed after chronic ethanol consumption. As discussed below, the induction of cytochrome P-450-mediated drug metabolism by ethanol ingestion may have considerable physiological implications.

As briefly mentioned above, the microsomal ethanol-oxidizing activity can be only partly attributed to the peroxidatic function of contaminating catalase. On the other hand, ethanol does not behave like a "conventional" cytochrome P-450 substrate. This becomes particularly clear when its interaction with cytochrome P-450 is examined, since there is no spectral evidence for the formation of an enzyme-substrate complex. In contrast, ethanol produces a reverse type I spectral change when added to the hemoprotein at high concentrations (Cinti et al. 1973; Rubin et al. 1971). Moreover, the metabolism of other drugs by cytochrome P-450 is inhibited mostly when ethanol is present at high concentrations and either in a competitive or in a noncompetitive manner (Cinti et al. 1973; Rubin and Lieber 1968; Schüppel 1971). With these observations in mind, it is particularly interesting to consider the recent proposal by Cederbaum and his colleagues (1978), that the catalase-independent microsomal oxidation of ethanol may be mediated by the generation of hydroxyl radicals. This theory is supported by the fact that ethanol is a well-known scavenger of hydroxyl radicals and that hydroxyl radicals are apparently generated during NADPH oxidation by the microsomal system (Cohen and Cederbaum 1979). The latter may occur in the presence of a combination of superoxide radical and hydrogen peroxide (the Haber-Weiss reaction) or by ferrous ion-catalyzed decomposition of H_2O_2 (the Fenton reaction) (Cederbaum et al. 1978). In both cases the generation of hydroxyl radicals depends on the access to H_2O_2, which is a known product of the microsomal NADPH oxidase reaction which, in turn, is markedly enhanced after ethanol consumption (Lieber and DeCarli 1970). A similar induction of microsomal NADPH oxidase by phenobarbital administration may explain the stimulation of microsomal ethanol oxidation observed after treatment with this type of enzyme inducers (Lieber 1975).

The inability to affect hepatic ethanol metabolism significantly by administration of catalase inhibitors has been taken to indicate that the peroxidatic activity of catalase plays a minor role in the oxidation of ethanol in vivo. Although this may be true under normal conditions, the situation could change under conditions of enhanced intracellular H_2O_2 generation. In experiments with isolated hepatocytes, both menadione and glycolate have been shown to stimulate ethanol oxidation markedly (Schulman et al. 1977); menadione generates H_2O_2 in the cytosolic compartment by autooxidation following enzymatic reduction, whereas glycolate stimulates peroxisomal generation of H_2O_2 by glycolate oxidase.

The autooxidation of cytochrome P-450 is one of the reactions contributing to the intracellular generation of H_2O_2. Most probably this reaction involves the dissociation of oxy-cytochrome P-450 to yield O_2^- which subsequently dismutates to generate H_2O_2 (figure 4; Kuthan et al. 1978). The reaction is enhanced by various induction regimens and markedly stimulated in the presence of several drug substrates (Hildebrandt et al. 1975). Recently it has been possible to show enhanced H_2O_2 generation by this mechanism during drug metabolism in isolated hepatocytes (Jones et al. 1978), and it appears conceivable that the stimulation of hepatic ethanol oxidation observed in the presence of drugs which also stimulate H_2O_2 generation could at least in part be due to an H_2O_2-dependent pathway for ethanol oxidation. The latter could involve either the peroxidatic activity of catalase or a hydroxyl radical-mediated reaction (Cederbaum et al. 1978).

Effects of Ethanol on Drug Metabolism

Ethanol may affect drug biotransformation by phase I as well as phase II reactions, and the effect may be either stimulatory or inhibitory. As mentioned above, the discovery of the induction of the hepatic microsomal cytochrome P-450-linked monooxygenase system by ethanol administration was soon followed by observations indicating enhanced rates of metabolism of a variety of drugs in liver microsomes from ethanol-treated experimental animals (Lieber 1975). These findings strongly suggest that the stimulation of drug metabolism in vivo observed after chronic ethanol consumption is attributable to the induction by ethanol of the hepatic microsomal cytochrome P-450 system.

The discovery of the involvement of cytochrome P-450 in the microsomal oxidation of ethanol also suggested that the well-known inhibition of microsomal drug metabolism by ethanol in vitro could be due to competition for cytochrome P-450. Although this seems to be a very reasonable hypothesis, it still lacks firm experimental support. The effect of ethanol on the hemoprotein seems to be to release endogenously bound substrate and thereby to convert more of the hemoprotein to its low-spin form (Cinti et al. 1973). In accordance with these observations, the inhibitory effect of ethanol on cytochrome P-450-linked drug monooxygenation would be attributed to the interference by ethanol with the binding of drug substrates to cytochrome P-450 rather than to a strict competition for metabolism through a common catalyst. Other experiments show, however, that the "binding" spectrum of ethanol to microsomes persists despite repeated washes with ethanol (Lieber, personal communication).

The activities of several phase II drug biotransformation reactions are influenced by the availability of the cofactor required for the reaction. Sulfate conjugation, in particular, appears often to be limited by the access to activated sulfate (Slattery and Levy 1977). Similarly, the formation of glucuronic acid conjugates depends on the continuous synthesis of activated glucuronic acid, i.e., UDP-glucuronic acid (Moldéus et al. 1979). As shown in figure 5, the synthesis of UDP-glucuronic acid requires UTP and glucose-1-phosphate and occurs by a two-step reaction sequence, whose latter part consists of the conversion of UDP-glucose to UDP-glucuronic acid catalyzed by UDP-glucose dehydrogenase. Like alcohol dehydrogenase-mediated ethanol oxidation, this reaction depends on the availability of NAD^+ and is inhibited by any shift in the redox level of the NAD^+/NADH couple toward the reduced state. The latter occurs during hepatic ethanol oxidation, and this is most probably the reason for the observed inhibition of the rate of glucuronidation of various drugs during ethanol metabolism by isolated hepatocytes (Moldéus et al. 1978). This conclusion is further supported by the finding that ethanol inhibition of drug glucuronidation in this system is abolished in the presence of 4-methylpyrazole. A similar dependency of ethanol oxidation and drug glucuronidation upon cytosolic NAD^+ concentration, or rate of NADH oxidation to NAD^+, may explain the effect of calcium ions on these processes in isolated hepatocytes (Andersson et al. 1979).

An opposite effect of ethanol metabolism on a phase II drug biotransformation reaction has been reported by Olsen and Mørland (1978). These authors observed that the rate of drug acetylation increased during ethanol metabolism in both healthy volunteers and isolated rat hepatocytes. Sulphadimidine was used as a model drug for acetylation in this study, and it was concluded that the stimulatory effect by ethanol was probably due to an increased concentration of acetyl coenzyme A during hepatic ethanol metabolism; acetyl coenzyme A is the acetate donor in the N-acetyltransferase reaction.

Conjugation with glutathione offers a major protective mechanism against toxic effects of various electrophilic drug metabolites. It is therefore of particular interest that Viña and collaborators (1980) recently have demonstrated a decrease in concentration of glutathione in isolated hepatocytes incubated with ethanol. This effect was maximal at 20 mM ethanol and was ascribed to the formation of an adduct between glutathione and acetaldehyde generated by the oxidation of ethanol. Possible toxicological implications of this phenomenon are discussed below.

From the discussion above it is apparent that several drug biotransformation reactions can be modified by ethanol via effects on either the catalyst involved or cofactor required in the reaction. Whereas chronic ethanol ingestion usually leads to stimulated drug biotransformation through enzyme induction, the acute effect of alcohol on drug biotransformation may be either stimulatory or inhibitory and most often mediated by a corresponding effect on the cofactor level. It is obvious that these metabolic interactions may have physiological as well as toxicological implications.

Physiological and Toxicological Implications of Metabolic Interactions Between Ethanol and Drugs

Research during the last two decades has revealed the existence of biochemical mechanisms which may explain physiologically important metabolic interactions between alcohol and various drugs. There is little doubt, for example, that the induction of the microsomal cytochrome P-450-linked monooxygenase system by ethanol is responsible for the increase in drug metabolism often observed after chronic alcohol consumption. Similarly, the inhibition by ethanol of cytochrome P-450-mediated drug oxidations in isolated liver microsomes offers a plausible explanation for the decreased rate of drug biotransformation after acute ethanol administration in vivo. The stimulation of drug acetylation and inhibiton of drug glucuronidation during ethanol metabolism provide additional examples of metabolic interactions between ethanol and drugs of potential physiological importance.

Alcohol has long been known to potentiate the hepatotoxic effect of certain drugs and other exogenous compounds (Strubelt 1980). This potentiating effect was originally observed with carbon tetrachloride, but has since been demonstrated also with a number of other foreign chemicals including chloroform, thioacetamide, dimethylnitrosamine, and aflatoxin B_1. Of particular clinical interest is the finding that the hepatotoxicity of the widely used analgesic drug acetaminophen is increased after ethanol ingestion (Emby and Fraser 1977). In view of the discussion above, it is apparent that several of the biochemical mechanisms thought to be important for the metabolic interactions between ethanol and drugs could also contribute to the potentiation by ethanol of the hepatotoxicity of acetaminophen and related drugs. These include induction of cytochrome P-450-mediated drug oxidation by chronic ethanol consumption as well as inhibited drug glucuronidation and enhanced glutathione utilization during ethanol metabolism.

Acetaminophen is activated to a hepatotoxic product by cytochrome P-450-mediated oxidation (Mitchell et al. 1973), and this reaction is markedly stimulated by various induction regiments including ethanol ingestion (Moldéus et al. 1980; Teschke et al. 1979). In contrast, formation of the glucuronic acid conjugate of acetaminophen is a detoxifying pathway, which also acts to lower the amount of drug available for metabolic activation by cytochrome P-450. Glutathione conjugation, finally, is the major protective mechanism against the hepatotoxic effect of the electrophilic acetaminophen metabolite formed in the cytochrome P-450-catalyzed reaction. Obviously, the known effects of ethanol on the reactions involved in acetaminophen metabolism will all act to potentiate the hepatotoxicity of this drug. Similar relationships may apply to the interaction between alcohol and several other hepatotoxic agents.

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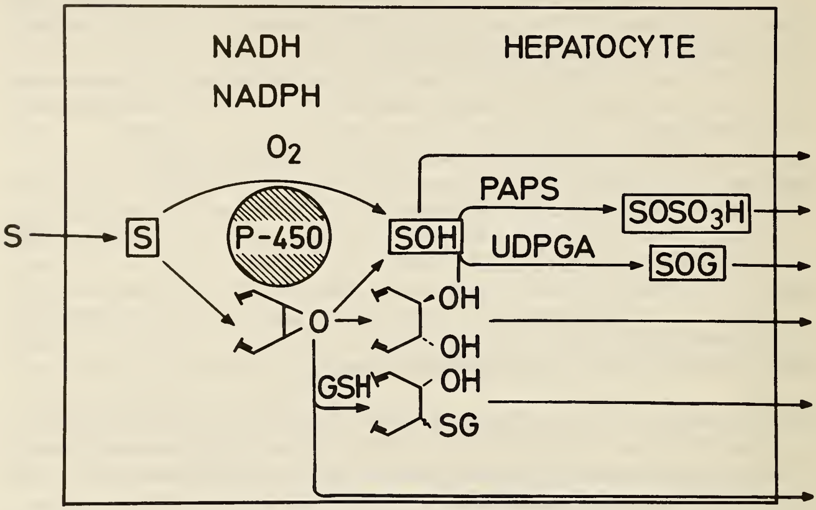
Figure 1. Biotransformation of Drugs by Oxidation and Conjugation in the Liver



S means substrate; SOH, hydroxylated product; SOG, glucuronide; GSH, glutathione, reduced form; SG, glutathione conjugate; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; UDPGA, uridine-5′-diphosphoglucuronic acid.

Figure 2. Oxidation of Ethanol to Acetaldehyde by Alcohol Dehydrogenase (A), the Catalase - H_2O_2 System (B), and the Microsomal Cytochrome P-450-Linked Monooxygenase System (C)

A.

$$CH_3CH_2OH + NAD^+ \xrightarrow[\text{Alcohol dehydrogenase}]{} CH_3CHO + NADH + H^+$$

B.

$$NADPH + H^+ + O_2 \xrightarrow[\text{Cytochrome P-450}]{} NADP^+ + H_2O_2 \qquad \text{(a)}$$

$$CH_3CH_2OH + H_2O_2 \xrightarrow[\text{Catalase}]{} CH_3CHO + 2H_2O \qquad \text{(b)}$$

C.

$$CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow[\text{Cytochrome P-450}]{} CH_3CHO + NADP^+ + 2H_2O$$

Figure 3. **Reaction Sequence Involved in Cytochrome P-450-Mediated Drug Monooxygenation**

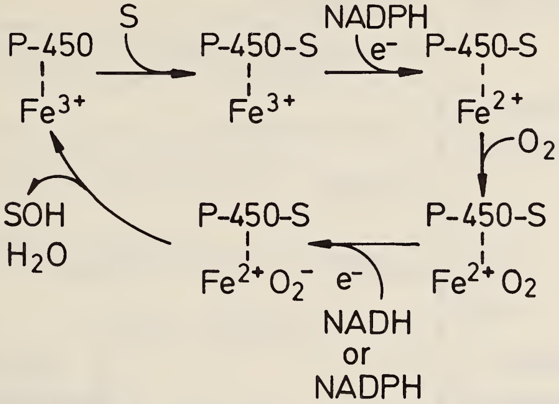


Figure 4. **Mechanism Proposed for Generation of Superoxide and Hydrogen Peroxide by the Cytochrome P-450-Linked Monooxygenase System**

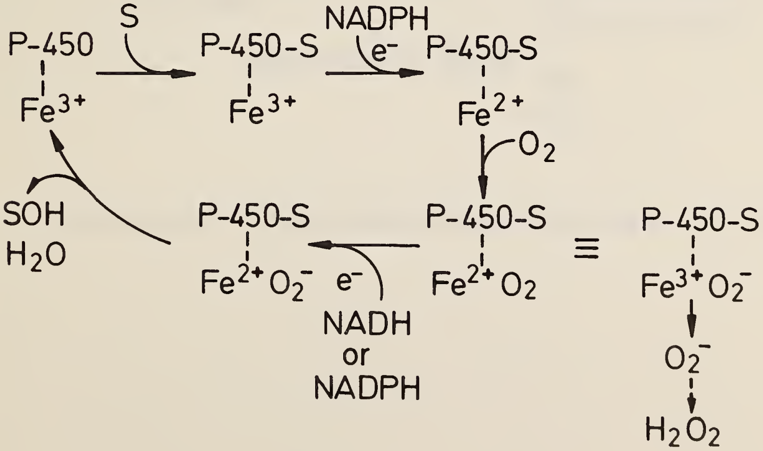
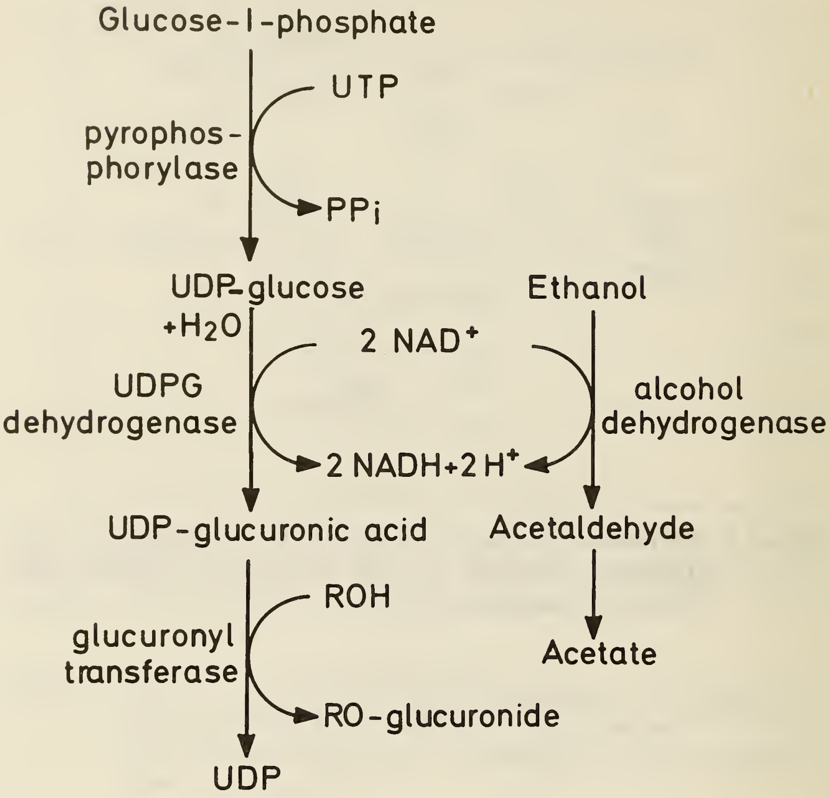


Figure 5. Interrelationship Between Ethanol Oxidation and Drug Glucuronidation in Liver



UDPG means uridine-5′-diphosphoglucose; ROH, hydroxylated drug metabolite.

Table 1. Metabolic Interactions Between Ethanol and Drugs

1. Effects of drugs on ethanol oxidation

    A. By alcohol and aldehyde dehydrogenases
        a. Inhibition of alcohol and aldehyde dehydrogenases
        b. Stimulation of reoxidation of NADH

    B. By microsomal ethanol-oxidizing system (MEOS)
        a. Competition for cytochrome P-450
        b. Induction of cytochrome P-450

    C. By catalase - H_2O_2 system
        a. Stimulation of intracellular H_2O_2 production

2. Effects of ethanol on drug metabolism

    A. By phase I reactions
        a. Interference with drug binding and metabolism by cytochrome P-450 system
        b. Induction of cytochrome P-450 system

    B. By phase II reactions
        a. Interference with UDP-glucuronic acid synthesis
        b. Stimulation of drug acetylation
        c. Ethanol-induced GSH consumption

Discussion of Paper by Orrenius

Esteban Mezey

Professor Orrenius has given us a very comprehensive description of the diverse and complex mechanisms of interactions between ethanol and drugs. We have made great strides from the simplistic notions of only a few years ago that inhibition of microsomal drug metabolism by ethanol was principally due to the binding of ethanol to cytochrome P-450, while enhancement of drug metabolism after chronic ethanol administration was caused by increases in cytochrome P-450. It has become apparent that numerous factors, e.g., the increase in reduced redox state, changes in the affinity of cytochrome P-450 for substrates, and the physico-chemical characteristics of microsomal membranes, may be important determinants of the alterations in drug metabolism observed after the administration of ethanol. Also, the effect of ethanol in inhibiting microsomal drug metabolism may not be a direct one, but may be mediated by the release of hormones that follow ethanol administration. In a recent study (Chung and Brown 1976), the known inhibition of hexobarbital hydroxylase activity produced by acute ethanol administration was not found in adrenalectomized animals, and corticosterone was found to be a competitive inhibitor of the enzyme.

The importance of the effect of ethanol on drug metabolism is not only that it can cause variations on the blood levels of the drugs and hence their therapeutic effectiveness at a given dosage of the drug, but by altering their disposition can lead to an increased formation of toxic metabolites. An example of the latter is the enhancement of acetaminophen toxicity by alcohol mentioned by Professor Orrenius.

I would now like to briefly discuss known effects of ethanol on nonmicrosomal pathways of drug metabolism. Professor Orrenius already mentioned that the effect of ethanol on inhibiting glucuronidation may be related to decreased formation of UDP-glucuronic acid as a result of the increased NADH/NAD$^+$ ratio occurring during ethanol metabolism. UDP-glucuronic acid, which is the donor of the conjugation reaction, is formed from UDP glucose, a reaction that is catalyzed by the NAD$^+$ dependent enzyme UDP-glucose dehydrogenase. In addition, the acute administration of ethanol has been shown to result in increases in the reduction of chloral hydrate and in the acetylation of sulphadimidine. The increased NADH/NAD ratio occurring during ethanol metabolism enhances reduction of chloral hydrate to its active metabolite trichloroethanol, a reaction catalyzed by alcohol dehydrogenase (Sellers et al. 1972). Olsen and

Mørland (1978) from Norway demonstrated an increased acetylation of sulphadimidine after the acute administration of ethanol in man. The effect was observed in both slow and rapid acetylators. The acetylation reaction takes place in the cytoplasm of the liver cell. The enhanced acetylation may be due to an increase in acetyl coenzyme A originating from acetate produced during ethanol metabolism. Acetyl coenzyme A is the donor of the N-acetyltransferase reaction which acetylates drugs. The increased acetylation demonstrated for sulphadimidine may also apply to other drugs such as isoniazid, resulting in enhanced production of toxic metabolites. It may account for the apparent increased occurrence of fulminant hepatitis due to isoniazid noted in alcoholic patients (Levin and Moodie 1974).

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Pathogenesis of the Fetal Alcohol Syndrome*

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Introduction

The teratogenic potential of maternal ethanol consumption has been suspected since antiquity, yet only in the past 10 years have in-depth clinical and preclinical studies been undertaken. The term fetal alcohol syndrome (FAS), as coined by Jones et al. (1973), has been applied to a constellation of specific craniofacial and developmental abnormalities seen in offspring of women who drank chronically during pregnancy (Hanson et al. 1976). The most commonly cited abnormalities are prenatal and postnatal growth deficiencies, developmental delay, microcephaly, short palpebral fissures, midfacial hypoplasia, joint abnormalities, cardiac defects, fine motor dysfunction, and renal abnormalities (Hanson et al. 1976). Neuropathological studies have uncovered a number of developmental errors possibly related to alterations in neuronal and glial cell migration (Peiffer et al. 1979).

The above defects are those associated with maternal consumption of large quantities of alcoholic beverages throughout gestation (Hanson et al. 1976; Jones et al. 1973) in humans. More recent studies have provided evidence that (a) moderate to high ethanol consumption during the second trimester significantly increases the chances of miscarriage (Harlap and Shiono 1980), (b) moderate drinking throughout pregnancy is associated with growth deficits and fetal abnormalities (Hanson et al. 1978; Little 1977), and (c) women with heavy drinking histories who abstain during pregnancy tend to bear underweight infants but to a lesser degree than if they had continued drinking (Little et al. 1980).

Animal studies have revealed that exposure of rodents to ethanol, in utero, results in prenatal and postnatal growth deficiencies (Abel 1978; Abel and Dintcheff 1978; Henderson and Schenker 1977; Henderson et al. 1979; Schwetz et al. 1978; Tze and Lee 1975), increased fetal death (Henderson et al. 1979; Randall and Taylor 1979;

NOTE: Figures and tables appear at end of paper.

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Tze and Lee 1975), and in increased incidence of congenital abnormalities (Kronick 1976; Randall et al. 1977; Schwetz et al. 1978; Tze and Lee 1975). Recent studies have indicated that the rate of occurrence of fetal morphological abnormalities is related to maternal blood ethanol levels (Randall and Taylor 1979). Thus, evidence exists that ethanol, even in moderate doses at limited time points during gestation, may exert fetotoxic effects. However, the mechanism(s) by which maternal ethanol consumption induces these abnormalities are not yet clear. Nevertheless, there is strong experimental evidence that ethanol exposure in utero can alter basic biosynthetic mechanisms required for normal fetal growth and development. Of primary importance to fetal growth are the systems which enable the developing organism to accrue protein and to acquire the nutrients necessary to maintain normal cellular growth. The following discussions will focus on the mechanisms (table 1) by which maternal ethanol consumption may induce fetal growth and developmental deficits.

The information presented in the following pages has been largely covered in a manuscript entitled "Fetal Alcohol Syndrome: Overview of Pathogenesis" published in *Pharmacology, Biochemistry and Behavior.*

Pathogenic Factors

The Role of Ethanol and Its Metabolite, Acetaldehyde

Alcohol in adults is rapidly metabolized to acetaldehyde and then to acetate. It is therefore reasonable to consider the roles of ethanol per se and its metabolites in the fetotoxic effects associated with maternal ethanol ingestion. Data in this regard are scarce; however, several studies have indicated that alcohol in the absence of acetaldehyde may be teratogenic. These are as follows: (a) In a recent study by Brown et al. (1979), rat embryos exposed to ethanol (3.0 mg/ml) in vitro for 48 hours showed decreases in total weight and in DNA and protein content. This occurred in the absence of ethanol metabolism, implying a direct effect of ethanol per se; (b) t-butanol, which is not metabolized to acetaldehyde, has been reported to have a similar deleterious effect on newborn mice as ethanol (Daniels and Evans 1980).

One study has attempted to determine the effect of acetaldehyde alone on the fetus. O'Shea and Kaufman (1979) administered 0.1 ml of 1 percent and 2 percent acetaldehyde intravenously to 25 g pregnant mice on days 7, 8, and 9 of gestation. The incidence of resorptions was significantly greater following acetaldehyde administration, the length and weight of these embryos decreased, and their protein content likewise diminished. For most of these parameters, the effect of acetaldehyde was dose-dependent. An apparent increase in neurological and cardiovascular anomalies also was noted in the acetaldehyde group. It should be appreciated that the dose of acetaldehyde injected was large, that blood and tissue levels of acetaldehyde were not monitored in the study, and that there is suggestive evidence that acetaldehyde at 100 nmol/ml maternal blood concentrations does not readily pass into the rat fetal circulation (Kesaniemi and Sippel 1975). In addition, only very low activity of acetaldehyde dehydrogenase has been found in rat placenta at 15 and 20 days of gestation (Sjoblum et al. 1978). It follows that if acetaldehyde alone is to produce a fetotoxic effect, it would not be related to a direct effect on fetal tissues, but rather due to a secondary insult, such as on placental function. The available data suggest that acetaldehyde may have teratogenic potential, but more quantitation in this area is needed. At present there is no information about the effect of acetate on the fetus. A combined toxic effect of alcohol and acetaldehyde has been demonstrated on cardiac mitochondrial protein synthesis (Burke and Rubin 1979).

Mutagenicity

The possibility that ethanol may have mutagenic effects has been examined and the data have recently been reviewed by Obe et al. (1979). The observations in animals are as follows: (a) No testicular chromosomal damage has been noted in Wistar rats exposed to 10 percent ethanol for up to 70 days (Kohila et al. 1976) and no chromosomal damage detected in Swiss mice drinking up to 40 percent ethanol for 27 days. (b) An increased incidence of dominant lethals was found by Badr and Badr (1975) when male CBA mice were given alcohol. However, this was not corroborated with similar ethanol doses in CD-1 and Swiss mice and in Wistar rats (Obe et al. 1979). Also, ethanol did not enhance the induction of dominant lethals by X-rays or methylmethane sulfonate (Obe et al. 1979). (c) Ethanol in vitro did not cause chromosomal damage in mouse ascites tumor cells and Chinese hamster ovary cells (Obe et al. 1979; Schoneich 1966). Studies of ethanol as a mutagenic agent in man show that (a) ethanol added in vitro to human lymphocytes and HeLa cells had no effect on chromosomal structure; (b) in alcoholic patients there was a higher incidence of chromosomal aberrations (i.e., ring chromosomes, chromatid exchanges) than in controls (Obe et al. 1979); and (c) lymphocyte chromosomes of children with FAS were normal, suggesting no direct ethanol-induced chromosomal impairment (Obe and Majewski 1978).

In conclusion, the animal data show little evidence of adverse influence of paternal drinking on the fetus or of a mutagenic effect of ethanol. Studies in man show that in alcoholics there does tend to be more chromosomal damage. This may be due to alcohol metabolites (i.e., acetaldehyde) or other mutagenic contaminants of alcoholism (i.e., poor nutrition, smoking, etc.). At present, there is no definitive evidence for paternal contribution to FAS.

The Role of Impaired Nutrition

Alcoholism is often accompanied by malnutrition; therefore, the possibility must be considered that impaired nutrition contributes to FAS. Specific examples of nutrient deficiency which can affect growth and development or cause congenital anomalies include lack of protein, of vitamins such as folate or minerals such as zinc or magnesium (Rosett 1979). Impaired protein intake, especially late in pregnancy, may result in underweight babies (Naeye et al. 1973), and folate deficiency (induced by folate antagonists or dietary deficiency) can cause fetal resorption, stillbirths, and congenital anomalies in the rat and perhaps human fetal malformations as well (Lindenbaum 1974; Sullivan 1967). Zinc deficiency also has been shown to cause fetal malformations (Hurley et al. 1971; Hurley and Swenerton 1966), and magnesium has a potentially important role in fetal growth since it stabilizes nucleic acids and is involved in other phases of protein synthesis (Rosett 1979). These nutritional deficits may be induced by reduced food intake and decreased absorption and/or impaired utilizations of nutrients. In addition maternal ethanol intake may interfere with maternal-fetal nutrient exchanges (Henderson et al. 1980).

Information available concerning the role of nutritional impairment in FAS is as follows: (1) With respect to humans, prospective evaluations by history of alcoholic women with FAS children have found no selective impairment of nutrient intake (Rosett et al. 1976). Two further arguments against prenatal undernutrition as the teratogenic source are: FAS babies are usually short for weight at birth (Hanson et al. 1976) and show no postnatal catchup phase with subsequent adequate nutrition (Jones et al. 1973) as with a nutritional deficit. (2) In experimental animals, developmental defects have been induced by only a few high doses of ethanol (Henderson et al. 1979) which are not likely to induce malnutrition. Also, growth deficits have been induced in offspring of rodents consuming ethanol chronically even when nutritional intake has been normalized by pair feeding (Henderson et al. 1979; Abel and Dintcheff 1978). Finally, the demonstration of impaired embryonic development in in vitro culture systems (devoid of maternal influences) exposed briefly to alcohol implies a direct toxic effect of alcohol independent of generalized malnutrition (Brown et al. 1979). It should be noted that the preceding studies do not exclude ethanol-induced changes in cellular uptake of specific nutrients or in altered assimilation of these nutrients. (3) Normal levels of folate have been found in most studies of rats chronically exposed in utero to ethanol (unpublished personal data) and exogenous folate did not prevent the development of ethanol-induced fetal development deficits (Lin et al. 1978). (4) In our studies of pregnant rats chronically exposed to alcohol with resultant adverse effects on the fetus, normal levels of zinc were found in the brain and femur of the adult animals and femur and muscle of fetal rats (Henderson et al. 1979). Likewise, normal fetal levels of magnesium were noted by Abel following chronic alcohol exposure (Abel and Greizerstein 1979). These data, therefore, suggest that a deficiency of these minerals is not primarily responsible for the experimental FAS, but more extensive studies of trace minerals in other animal models of this syndrome and especially in prospective human surveys are indicated.

In conclusion, it would appear that alcohol per se can cause FAS and that malnutrition is not an essential component. Decreased intake or assimilation of key vitamins or minerals or other nutrients could contribute importantly to this syndrome, as could the effects of excess alcohol intake on placental transport of such substances. These areas have had little assessment so far either in animal models or in human FAS.

Abnormal Neurotransmitter Status

Since children with FAS show intellectual and behavioral manifestations associated with brain dysfunction, it seemed reasonable to postulate that excess alcohol intake may alter the cerebral neurotransmitter balance and thus cause some of these deficits. The effects of acute and chronic alcohol intake, as well as its withdrawal, on cerebral neurotransmitters in adult animals have been extensively reviewed (Noble and Tewari 1977; Schenker 1980). The data are complex and controversial regarding both actual results and their physiological significance. In a study of FAS, chronic maternal ethanol intake failed to alter the net levels of serotonin or norepinephrine in fetal or neonatal whole brain (Rawat 1977), although in one study tyrosine hydroxylase activity in the brain of newborn rats exposed in utero to ethanol was increased (Branchey and Friedhoff 1973) and that of dopamine β-hydroxylase was decreased (Detering et al. 1980). We are unaware of studies on regional cerebral catecholamine turnover in such animals. In the adrenal glands of newborn rats there was evidence of delayed maturation of catecholamine stores (Lau et al. 1976), but the immediate cause and physiological significance of this are not clear. Other cerebral neurotransmitters reported to be altered in the ethanol-exposed fetal and newborn rats consist of increased histamine (Rawat 1980), γ-aminobutyric acid, and glutamic acid, and a fall in acetylcholine (Rawat 1976). The latter measurement, however, is suspect since the concentrations are low and alcohol is believed to induce an increase in brain acetylcholine (Parker et al. 1978).

The overall significance of these few reported changes in brain neurotransmitters is uncertain. The studies were generally carried out in whole brain, in many instances a possible confounding nutritional impairment could not be ruled out, turnover data are absent, and the functional relevance of the changes observed is unknown.

Hormonal Changes

Various hormonal changes involving corticosteroid, sex hormones (Kakihana et al. 1980), and glucagon have been reported following alcohol intake. The subject is discussed in detail in a recent review (Kakihana and Butte 1979). This brief commentary will only consider possible changes in growth hormone homeostasis since alterations in this hormone have been linked closely to the pathogenesis of the FAS.

One concept is that growth hormone output from the hypothalamus may be decreased in the fetus exposed to ethanol, that ornithine decarboxylase (ODC) activity, which is "regulated" by growth hormone (in addition to many other factors), may be decreased in FAS, and this in turn may diminish the synthesis of polyamines which may influence protein synthesis and thus fetal growth (Thadani and Schanberg 1979). This concept is supported by the observations that (a) ethanol given acutely to animals induces a decrease in serum growth hormone levels and a fall in brain and heart ODC activity (Thadani and Schanberg 1979); and (b) chronic alcohol intake in animals alters the normal developmental pattern of growth hormone and ODC activity in a complex manner dependent on the quantity and duration of alcohol intake and the tissue studied (Thadani et al. 1977*a*,*b*). While these observations suggest some correlation between ethanol intake and growth hormone levels and effects, it should be emphasized that in nine children with FAS, growth hormone release in response to insulin and arginine infusion was unimpaired or slightly increased (Root et al. 1975; Tze et al. 1976). In addition, growth hormone and ODC are influenced by stress, cold, and other nonspecific variables (Kakihana and Butte 1979). It should also be noted that the relationship of this hormone to fetal growth has not been established. Thus, the specificity and causal importance of growth hormone-ODC changes in FAS are uncertain and require further study.

Protein Synthesis

Most, if not all, of the fetal defects associated with maternal ethanol consumption can be related to deficits in growth of embryonic or fetal tissues. Growth is a complex process which is reflected in the ability of an organism to accrue protein. Several studies have indicated that, as in adult tissues, ethanol exposure can alter some parameters of the protein synthetic machinery in the fetus.

Evidence exists that in the adults, alcohol given both acutely (Jarlstedt and Hamberger 1972; Kuriyama et al. 1971) and chronically (Jarlstedt and Hamberger 1972; Tewari and Noble 1971) depressed protein synthesis in brain. Similar findings have been reported for liver (Hofmann and Hosein 1978; Morland and Bessesen 1977; Perin et al. 1974) and the heart (Schreiber et al. 1972) using both in vivo and in vitro systems. Both ethanol and acetaldehyde in vitro have been shown to depress protein synthesis, but the precise relative roles of these substances in in vivo situations is still uncertain. A number of studies have attempted to assess the specific steps in protein synthesis where ethanol exerts its effects. Of these, an impairment of animoacyl-tRNA synthetase for various specific amino acids and decreased transfer of mRNA from the nucleus to the cytoplasm seem best established, but others may likewise contribute. Chronic alcohol ingestion has also been reported to cause an increased turnover of RNA in rat brain mitochondria (Tewari et al. 1978). This subject has been recently reviewed (Tewari and Noble 1979).

It should be emphasized that although the majority of studies have shown significant impairment of protein synthesis in various tissues following alcohol administration, some dissents remain (Kuriyama et al. 1971). Some of the difficulties in this field can be attributed to differences in species, tissues used, doses of alcohol employed, and duration of the studies. Other problems in the interpretation of some data are (a) lack of information regarding in vivo ethanol levels; (b) failure to quantify endogenous amino acid precursor pools which could falsely depress incorporation rates of labeled precursor amino acids into protein; and (c) lack of information as to the role of alcohol-induced hypothermia on in vivo and even possibly subsequent in vitro protein synthesis.

Most of the studies on the effect of alcohol on protein synthesis in the fetus and neonate have been carried out by Rawat (1975, 1976, 1979). Chronic maternal ethanol consumption (2-3 weeks on a liquid diet) was reported to induce numerous alterations in protein synthesis in the fetus and neonate. These consisted of (a) reduced ^{14}C-leucine incorporation into cerebral ribosomes from fetal rats and neonates (Rawat 1975), fetal and neonatal cardiac protein (Rawat 1979), and fetal and neonatal hepatic protein (Rawat 1976); (b) reduced ability of fetal brain pH 5 enzyme fraction to synthesize ^{14}C-leucine tRNA (i.e., decreased leucyl-tRNA synthetase activity) (Rawat 1975); and (c) decrease in total RNA and DNA in neonatal brain (Rawat 1975), of RNA in fetal and neonatal heart (Rawat 1979) and fetal liver, as well as of total ribosomal protein in fetal liver (Rawat 1976). Not all of these findings have been confirmed by others. For example, no consistent decrease in fetal brain, heart, kidney, or liver total RNA was noted with chronic alcohol intake by Henderson et al. (1979). Likewise, when fetal net protein synthesis was estimated using large doses of ^{14}C-valine, incorporation in vivo after chronic alcohol intake was unaltered if the study was done at low blood alcohol levels in the absence of ethanol-induced hypothermia (Henderson et al. 1980).

However, net protein synthesis in fetal brain, heart, kidney, and liver was significantly ($p < 0.05$) reduced by 20 to 25 percent if a single acute dose (4 g/kg) of ethanol was administered to these dams, lowering maternal body temperature by 2.3°C and raising blood ethanol levels to about 3.0 mg/ml. Concomitant hypothermia was found to be the major contributor to the observed depression in net protein synthesis in fetal brain and kidney but not in fetal heart or liver. Thus, it was concluded that chronic maternal ethanol intake may reduce protein synthesis in the fetal rat, but only when maternal blood ethanol levels are elevated or concomitant hypothermia occurs. In the above regimens, direct effects of ethanol and/or acetaldehyde on net protein synthesis in fetal heart and liver are implied (Henderson et al. 1980). It should be appreciated that, in this study, total protein fractions were estimated and that ethanol-induced changes in synthesis of specific protein moities might not have been detectable. In vitro experiments using fetal tissues and those obtained from neonates also indicated a number of alcohol-induced changes in protein-synthesizing machinery. These consisted of observations that (a) ethanol reduced ^{14}C-leucine incorporation into ribosomes and pH 5 enzyme fractions (Rawat 1975) and (b) prior in vivo ethanol exposure inhibited subsequent in vitro protein synthesis with fetal and neonatal ribosomes and pH 5 enzyme fractions obtained from liver (Rawat 1979). However, in vitro ethanol had no effect on fetal hepatic net protein synthesis (Rawat 1976).

It seems evident from these composite data that ethanol does inhibit protein synthesis in various adult as well as fetal and neonatal tissues. This could explain, at least in part, the decrease in growth in animals and children with FAS. On the other hand, much needs to be done to understand this subject. For example, most of the work on the brain has utilized the whole organ, yet it exhibits major heterogeneity not only in function but also in the rate of development. There may even be a difference in response based on cell type with the glial cells which grow more later in pregnancy affected to a greater degree (Tewari and Noble 1979). Furthermore, many studies have utilized newborn animals nourished by other dams previously fed alcohol or even continuing on such a regimen. The effect of such a maternal regimen or even of prior alcohol exposure of the fetus on its subsequent nutrition are uncertain. It is important to consider also that brain development in many animals does not correspond well temporally to that of man, with the rat maturing later and the guinea pig earlier during gestation. Such variations in development in relation to alcohol exposure may have a bearing on extrapolating data from experimental animals to man. Finally, in most instances, net protein synthesis is measured, and the formative and degradative processes usually have not been dissected. There are some data that alcohol not only decreases protein synthesis but also causes a decrease in protein degradation (Chapman et al. 1979). Thus, the diminished protein formation with ethanol may represent a reduction in net protein synthesis. The role of ethanol-associated hypothermia should be considered in future studies.

Maternal-Fetal Exchange of Nutrients

A relatively new area of study in the pathogenesis of ethanol-induced abnormalities concerns the effect(s) of ethanol and its metabolites on maternal-fetal exchange of nutrients vital to fetal growth. Placental transfer is known to be a major source of amino acids required for fetal protein synthesis and possibly for metabolic fuel (Young 1979; Gresham et al. 1972). Therefore, deficiencies in supplies of the amino acids to the fetus could affect the amount of protein accrued, impairing fetal growth. Studies in our laboratory have indicated that, in the rat, a single dose of ethanol (4 g/kg) can reduce uptake of valine by placental villous fragments by 40 percent, independent of ethanol-induced hypothermia (figure 1). Forty to 50 percent reductions in placental uptake of valine, cycloleucine, and α-aminoisobutyric acid were also observed in the in vitro uptake system, in placental fragments obtained from dams chronically exposed to ethanol (figure 2). In vivo studies completed to date indicate that both acute and chronic ethanol exposure reduce net maternal-fetal exchange of valine in vivo by 45 to 50 percent (Patwardhan et al. 1980). In order to determine whether ethanol or acetaldehyde is the causal agent, villous fragments were preincubated with either compound for 2 hours prior to the valione uptake studies. As indicated in table 2, in vitro ethanol exposure at concentrations as low as 1.0 mg/ml will significantly reduce placental valine uptake while a net level of 465 μM acetaldehyde is required to elicit an effect. This concentration of ethanol is definitely physiological while it is unlikely that placental acetaldehyde concentrations approach 465 μM (Westcott et al. 1980; Kesaniemi and Sippel 1975). Therefore, at least in the rat, ethanol is probably the agent responsible for the observed changes in placental valine uptake following acute ethanol intake.

The inhibition of placental valine uptake by chronic and acute maternal ethanol consumption may contribute to the established teratogenic potential of ethanol. The altered ability to accumulate an essential amino acid in vitro is further reflected in reduced maternal-fetal exchange observed in the preceding in vivo studies. This composite work implies that acute and especially chronic maternal ethanol consumption can induce defects in the transfer of a nutrient vital to fetal development. The functional significance of this phenomenon needs to be the focus of further studies.

Other Factors

Alcohol has multiple effects on the body. For example, it affects neuronal and other membranes, altering their chemical composition and physical state (Noble and Tewari 1977; Schoneich 1966) and it interferes with mitochondrial structure and function (Burke and Rubin 1979; Noble and Tewari 1977). Any of such effects of ethanol in the fetus may contribute to the pathogenesis of FAS, but the relationship at this time is too tenuous and speculative to warrant further discussion. Furthermore, alcohol given to the mother may cause fetal acidosis (Mann et al. 1975). The effects of such changes in fetal pH remain to be elucidated.

Conclusions Concerning Specific Biochemical Mechanism(s) of FAS

It is evident that the biochemical mechanism(s) of FAS are uncertain, even when the syndrome is defined very liberally to include growth and developmental derangements noted in fetal and neonatal animals exposed to ethanol. Probably the most established concept relates to interference of ethanol with protein synthesis and seems to involve various steps in the protein synthesis machinery. The best defined abnormality relates to impaired formation of aminoacyl tRNA. Regional and cellular brain protein synthesis in the fetus and neonate normally and after exposure to ethanol and its metabolites, at periods of development comparable to man, remain to be investigated. Although data are scanty, there is little evidence that a mutagenic effect of ethanol plays an important role in the FAS. Changes in hormonal (growth hormone) milieu and cerebral neurotransmitters may occur following exposure to ethanol, but the functional significance of such findings is yet to be determined.

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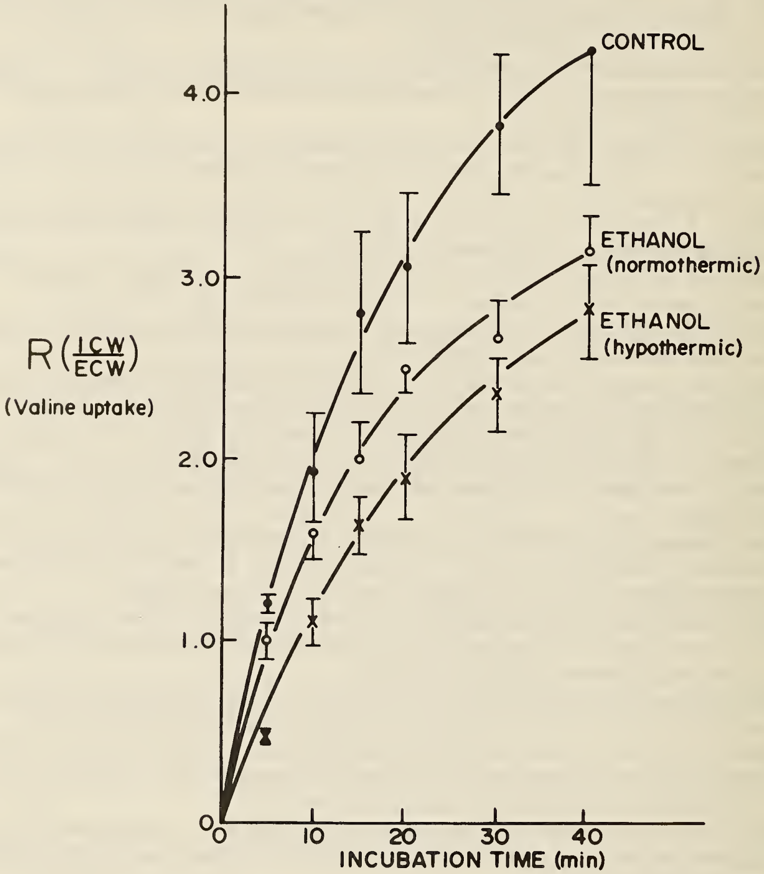
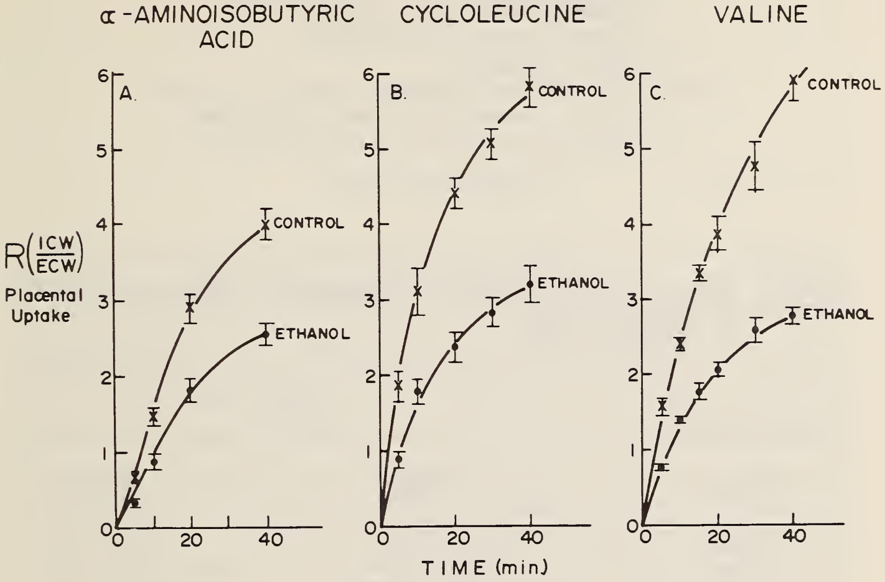
Figure 1. Effect of a Single Dose of Ethanol on Placental Valine Uptake Measured in Vitro



The vertical axis represents the ratio of accumulated ^{14}C valine in the intracellular water (ICW) to that in the extracellular water (ECW). The horizontal axis indicates the time of incubation of villous fragments following addition of ^{14}C valine (10^{-6}M). Ethanol (4 g/kg, 25 percent solution) or isocaloric dextrose was administered by intubation 2 hours prior to sacrifice on day 20 of gestation. Ethanol induced a 2°C drop in body temperature x——x, ethanol (hypothermic) as compared to normothermic controls (•——•, control). Twenty day pregnant dams labeled ethanol (normothermic) (○——○), were treated in a manner identical to ethanol (hypothermic) rats except that their body temperature was maintained at 38°C throughout the 2 hr ethanol exposure period. Placentas from each pregnancy were pooled and the resulting fragments divided into 4 incubation tubes, one for ICW determinations and 3 for val uptake studies. n = 12 dams and 126 placentas for control, n = 11 dams and 113 placentas for ethanol (hypothermic), n = 11 dams and 116 placentas for ethanol (normothermic). Vertical bars represent standard errors. The ethanol and control values differed statistically ($p < 0.05$).

Figure 2. Effect of Chronic Ethanol Consumption on Placental Amino Acid Uptake Measured in Vitro



The vertical axis represents the ratio of (A) ^{14}C α-aminoisobutyric acid, (B) ^{14}C cycloleucine, and (C) ^{14}C valine in the intracellular water (ICW) to that in the extracellular water (ECW). The horizontal axis indicates the time (min) following addition of the amino acid (10^{-6}M) to the incubation media. Rats were maintained on the Lieber-DeCarli liquid diet containing either 6 percent ethanol (chronic ethanol •——•) or isocalorically balanced maltose dextrins (pair-fed control x——x). At sacrifice on day 20 of gestation, blood ethanol levels averaged 1.8 mg/ml. Placentas from each pregnancy were pooled and the resulting fragments divided into 4 incubation tubes. For (A) n = 10 dams (90 placentas) for the pair-fed control rats and n = 10 dams (84 placentas) for ethanol-exposed rats; (B) n = 10 dams (104 placentas) for the pair-fed control rats and n = 10 dams (98 placentas) for ethanol-exposed rats; (C) n = 12 dams (98 placentas) for the pair-fed control rats and n = 12 dams (110 placentas) for the ethanol-exposed rats. Vertical bars represent standard errors. Ethanol and pair-fed control values differed statistically ($p < 0.05$).

Table 1. Pathogenic Factors in Fetal Alcohol Syndrome

1. Mutagenicity
2. Impaired nutrition
3. Abnormal neurotransmitter status
4. Hormonal changes
5. Impaired protein synthesis
6. Impaired maternal-fetal exchange of nutrients
7. Other factors (acidosis, membrane changes, etc.)

Table 2. Effect of Ethanol and Acetaldehyde on Placental Uptake of Valine in Vitro

Net Concentration**	$R\frac{(ICW)}{(ECW)}$* ± SE	
Ethanol	Ethanol	Control
3 mg/ml	1.20 ± 0.07***	1.63 ± 0.07
2 mg/ml	1.00 ± 0.08***	1.31 ± 0.08
1 mg/ml	1.25 ± 0.12***	1.61 ± 0.15
0.5 mg/ml	1.29 ± 0.10	1.50 ± 0.21
Acetaldehyde	Acetaldehyde	Control
620 μM	1.00 ± 0.09***	1.56 ± 0.14
465 μM	1.51 ± 0.14***	2.06 ± 0.27
232 μM	1.64 ± 0.19	1.55 ± 0.12

*$R\frac{(ICW)}{(ECW)}$ indicates the ratio of accumulated isotope in the intracellular water to that in the extracellular water (DPM/ml). Mean ± SE, n = 10 for each set of values.

**Placental fragments were exposed to ethanol or acetaldehyde for 2 hr of preincubation and throughout a 10 min incubation with ^{14}C val ($10^{-6}M$).

***Statistically significant ($p<0.05$).

The Influence of Ethanol and Acetaldehyde on Placental Protein Synthesis*

Sujata Tewari and Michael D. Christine

Introduction

Multiple biochemical and pathophysiological effects have been attributed to ethanol and its metabolites. Clinical and experimental studies have established ethanol as both a teratogenic and an embryotoxic agent. The consumption of alcoholic beverages or the therapeutic administration of alcohol to pregnant women is known to result in the rapid transfer of alcohol from the maternal to the fetal compartment. Ethanol therefore has the potential to alter the development of the embryo and fetus differently at the various stages of pregnancy. All of these observations establish the fetal alcohol syndrome (FAS) as a definite entity with specific morphological characteristics of prenatal and postnatal growth deficiency and mental retardation. Both prospective clinical studies and laboratory studies in experimental research, however, now support the view that there may be a wide range of "fetal ethanol" effects in the absence of the full syndrome (Rosett 1979). These fetal effects have been less extensively observed in offspring of women who drank heavily but not chronically during pregnancy, but more sporadically than for chronic drinkers.

Various investigations have suggested that acetaldehyde, the primary metabolite of ethanol, may be responsible for the toxic dependency syndrome producing the teratogenic properties following ethanol consumption (O'Shea and Kaufman 1979). In contrast to ethanol, there is a paucity of data on effects of acetaldehyde on the embryonic and fetal development. In the adult rat, ethanol is metabolized in the liver by oxidation to acetaldehyde, which is further oxidized to acetic acid. The two oxidations are catalyzed by the enzymes alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). Both of these enzymes are NAD dependent. In the developing rat, hepatic ADH activity can be detected just before birth followed by gradual increases to reach 82 percent of adult values at 47 days. The

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NOTE: Figures and tables appear at end of paper.

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ALDH I and II activity in the liver can be detected from day 15 of gestation with rapid increases at birth and reaching 80-90 percent of adult values at 47 days.

The placenta, which functions as a barrier as well as a bridge between the maternal and the fetal circulatory systems, is an organ with high metabolic activity that changes with gestational age (Villee 1962). The placenta, therefore, plays an important role during the extrauterine life of the organism. In the placenta, ADH and ALDH I and II activities when studied at 15 and 20 days of rat gestation were observed to be still lower at 20 days (Sjoblom et al. 1978). Human term placenta has been shown to have small ALDH activity (Kovri et al. 1977). While ethanol is known to pass freely across the placental membrane, the extent to which acetaldehyde does this is not known. All of these studies support that a very large proportion of unborn human fetuses are exposed to alcohol and probably its metabolites during gestation. Recent biochemical evidence shows that human placenta is rich in protein biosynthetic enzymes (Pennys and Muench 1974; de Venlay et al. 1978). The placental synthesis and secretion of protein and hormones have been established by incubation of placental tissues in vitro. However, information is not available on what effects, if any, that alcohol or acetaldehyde may have on cell-free protein synthesis by human placental ribosomes. This report investigates these issues. Experiments have been designed to precisely locate the specific steps of protein synthesizing systems which may be influenced by ethanol and acetaldehyde. The possible significance of these findings is discussed.

Materials and Methods

Chemicals

All reagents used chemicals of the highest grade obtainable and were made with double-deionized water. (^{14}C) leucine (specific activity 342 mC/m mole) was purchased from the Schwarz Bioresearch Company (Orangeburg, N.Y.). (^{14}C) phenylalanine (specific activity 0.05 mC/0.018 mg) and (^{14}C) phenylalanyl-tRNA (specific activities 0.12 mC/mg) were purchased from the New England Nuclear Company (Boston, Mass.). ATP and GTP were obtained from Sigma Chemical Company (St. Louis, Mo.).

Source of Placenta

Freshly delivered human placenta was obtained within 15 minutes of delivery from the University of California at Irvine Medical Center. The placenta was washed thoroughly with double-deionized water and only tissue from the maternal side of the placenta was used. The top 1 cm of the tissue was destroyed to avoid contamination.

Preparation of Placental Ribosomes and pH5 Enzymes Fraction

Following washing, the total weight of the placenta was obtained. A freshly obtained placenta weighed an average of 500 g. For the isolation of subcellular fractions, approximately 100 g of tissue was taken and washed in 100 ml portion of medium M (0.25 M sucrose, 0.1 M KCl, 0.04 M NaCl, 0.006 M 2-mercaptoethanol, 0.007 M Mg acetate, 0.02 M Tris, pH7.6). Placental ribosomes and pH5 enzymes fraction were prepared by the procedure described by Tewari and Noble (1971). Briefly, the placenta was homogenized in 1 volume of medium M followed by centrifugation at 15,000 g to remove a top layer of lipids and nuclear and mitochondrial fractions in the pellet. The postmitochondrial supernatant was subjected to high speed centrifugation at 105,000 g for 130 min; and 1 percent deoxycholate treatment yielded total ribosomes with the pH5 enzymes and soluble factors obtained.

Results

Properties of the Placental Protein Synthesizing System

The analysis of the placental subcellular fraction shows this tissue to be a rich source of ribosomes and pH5 enzymes-soluble fractions which exhibited high rates of in vitro protein synthetic activity (table 1). According to protein content determinations, each gram of human placenta will yield approximately 0.5 mg ribosomal protein fraction and 1.5 mg pH5 enzymes protein fractions. Thus, the amount of protein present in the pH5 enzymes precipitable fraction was three times as much as that of the ribosomal protein fraction in the human placenta. Both of these fractions were biologically active as demonstrated by the active incorporation of both (^{14}C) leucine and (^{14}C) phenylalanine into protein following the incubation in the presence of ATP and GTP at 37°C for 60 minutes. The incorporation of leucine occurred at a much higher rate than phenylalanine. Using these in vitro incubation conditions, approximately 70 $\mu\mu$ moles of phenylalanine and 800 $\mu\mu$ moles of leucine can be incorporated into protein by the placental ribosomal system from the postmicrosomal supernatant fraction (Tewari and Noble 1971). The ribosomal pellets were frozen immediately following isolation at −70°C.

Assays

Protein contents of ribosomal and pH5 enzymes precipitable fractions were determined by the method of Lowry et al. (1951).

Analysis of Cell Free Protein Synthesis

The in vitro incorporation of (^{14}C) phenylalanine or (^{14}C) leucine into hot TCA precipitable residue was measured according to previously established procedures (Tewari and Noble 1971).

In a separate experiment, it was determined whether the cell-free protein synthesis was dependent upon the time that the fractions were incubated. A time curve incubation study showed an active incorporation into protein for both amino acids by placental ribosomes up to 60 min (figure 1). Since aminoacylation of tRNA is a prerequisite for the incorporation of amino acids into protein, the placental pH5 enzymes fraction from humans have active leucyl-tRNA synthetase and phenylalanyl-tRNA synthetase. As in table 1, data further show a greater rate of incorporation for leucine than phenylalanine at all time points.

Effects of Ethanol and Acetaldehyde

Table 2 presents data in the presence and absence of 100 mM ethanol and 10 mM acetaldehyde in the incubation medium on the incorporation of (^{14}C) leucine and (^{14}C) phenylalanine into protein. These findings indicate that the incorporation of both leucine and phenylalanine was unaffected by the in vitro addition of ethanol (93 percent and 100 percent of standard activity, respectively). However, the addition of acetaldehyde markedly reduced the incorporation of both amino acids into protein when compared to the standard condition: The observed inhibition was somewhat greater with (^{14}C) leucine (45 percent) than (^{14}C) phenylalanine (70 percent of the standard activity).

The investigations were continued by examining the effects of ethanol and acetaldehyde on the ribosomal system by measuring the in vitro protein synthetic activity at different time periods. These data are given in figure 2. Figure 2A presents findings on the incorporation of (^{14}C) leucine into protein in the presence and absence of ethanol or acetaldehyde at different time points of incubation. Both under the standard conditions and in the presence of ethanol, the incorporation of (^{14}C) leucine into protein continued for at least 60 min and the ribosomal activity was unaffected by ethanol. However, the addition of acetaldehyde greatly reduced the protein synthetic activity at all time points. After acetaldehyde addition, no active in vitro protein synthesis occurred between 30 and 60 min. When the effects of ethanol and acetaldehyde were determined on the time-dependent incorporation of (^{14}C) phenylalanine into proteins, very similar results were obtained (figure 2B). The (^{14}C) phenylalanine incorporation was linear and unaffected (as in figure 2A) for at least 60 min for the ribosomal system in both the presence and the absence of ethanol. However, although the protein synthetic activity was inhibited at all time points in the presence of acetaldehyde, the placental ribosomal system did not stop incorporating (^{14}C) phenylalanine into protein after 30 min as it did with leucine in figure 2A. In fact, active incorporation continued to occur until 60 min in spite of significant acetaldehyde-induced reduction of the protein synthetic activity.

The Effects of Ethanol and Acetaldehyde on the in vitro Protein Synthesizing System Using (^{14}C) Phenylalanine and (^{14}C) Phenylalanyl-tRNA as Precursors: The Sites of Action

The above findings showed significant inhibition by acetaldehyde of in vitro protein synthesis which was unaffected by ethanol under the same experimental conditions. In an attempt to further delineate the effects of ethanol from acetaldehyde on protein synthesis, we have studied the availability of messenger RNA and peptide bond formation in the presence of these drugs. For these experiments we chose to use phenylalanine instead of leucine for the following reasons. First, we can use polyuidylic acid, or poly (U), a synthetic mRNA that specifically codes for phenylalanine; second, prelabeled (^{14}C) phenylalanyl-tRNA is available commercially and can be used in specific experiments instead of free phenylalanine as the precursors in the incorporation studies. The prelabeled (^{14}C) phe-tRNA can be used to bypass the aminoacylation steps of protein biosynthesis and thus focus on the formation of the peptide bond on polyribosomes.

When free (^{14}C) phenylalanine was used in the protein synthesis assay, the addition of poly (U) greatly increased the incorporation rate by more than 300 percent at 5 min and more than 800 percent at all other time points (figures 3 and 4). Ethanol again was without effect on the phenylalanine incorporation in the presence and absence of poly U (figure 3). As before, the addition of acetaldehyde was inhibitory to the phenylalanine incorporation both in the presence of endogenous mRNA and when the system was supplemented with poly U (figure 4).

With (^{14}C) phenylalanyl-tRNA as the chosen precursor, active conversion of (^{14}C) phenylalanine into ribosomal bound polypeptide chains was observed (table 3). The reaction was GTP dependent and proceeded in the absence of ATP. The addition of poly U was highly stimulatory to the transfer of radioactivity from (^{14}C) phe-tRNA to (^{14}C) polyphenylalanine (479 percent) and was unaffected by the presence of ethanol and acetaldehyde (479 percent and 497 percent, respectively). Ethanol and acetaldehyde also had no effect on the standard activities in the absence of poly U. Thus, the previously observed inhibition of protein synthesis induced by acetaldehyde was abolished by using (^{14}C) phe-tRNA rather than free (^{14}C) phenylalanine as the substrate. This is important, as it signifies the localization of acetaldehyde effects being between the reaction steps in the conversion of (^{14}C) phenylalanine to (^{14}C) phenylalanyl-tRNA by the phenylalanyl-tRNA synthetase.

When the total percentage transfer of radioactivity was calculated from the conversion of (^{14}C) phe-tRNA to ribosome bound (^{14}C) phenylalanine labeled peptide chain, only about 5 percent of the total radioactivity was transferred in the presence of endogenous mRNA (table 4). With the addition of poly U, the conversion to peptidyl bond formation was stimulated to reach 25 percent. Both ethanol and acetaldehyde had no effect on the percentage transfer of phenylalanine from (^{14}C) phe-tRNA to ribosome bound (^{14}C) polypeptide in either the presence or absence of poly U.

A final experiment was performed testing the effects of two different concentrations of ethanol and three concentrations of acetaldehyde on the transfer of (^{14}C) phenylalanine from (^{14}C) phe-tRNA to ribosomal bound polypeptides. Findings in table 5 describe the total counts transferred from (^{14}C) phe-tRNA to ribosomes during the peptide bond formation. As before with endogenous mRNA (table 4), only about 5 percent of radioactivity is recovered in polypeptides. The recovery or transfer process is again greatly augmented by the addition of exogenous mRNA. Ethanol at both concentrations was without effect on this process. Acetaldehyde at 1 mM and 10 mM had no effect while 100 mM was extremely inhibitory to the transfer process (0.45 percent). Even with the addition of poly (U) when 100 mM acetaldehyde was present, the conversion process remained extremely low (2.3 percent).

The last table (table 6) summarizes the results obtained on placental protein synthesis by adding 100 mM ethanol and 10 mM acetaldehyde using all three precursors: (^{14}C) leucine, (^{14}C) phenylalanine, and (^{14}C) phenylalanyl-tRNA. We found that 10 mM acetaldehyde was inhibitory only when the free amino acids leucine and phenylalanine were used as substrates under our conditions. When (^{14}C) phe-tRNA is used as the substrate wherein the aminoacylation steps are bypassed, the previously observed inhibition of protein synthesis by acetaldehyde is abolished and activities were restored to standard conditions.

Discussion

It is now well established that secretory proteins and polypeptides are synthesized by the developing human placenta. The placenta is a rich source of thyrotropin hormone releasing lactogen and alpha-feto protein (AFP). Elevated AFT blood levels in toxemic cases may reflect increased placental levels. Specific effects of toxins on placental protein synthesis, however, have not been widely studied. Observation by O'Shea and Kaufman (1979) demonstrated that the teratogenic and embryotoxic effects of acetaldehyde when administered intravenously to pregnant mice during the early postimplantation period were dose dependent; fetal abnormalities occurred in the CNS and seemed mainly to result from the failure of normal closure of the cranial and caudal regions of the neural tube. However, information is lacking on acetaldehyde effects on the placenta. Furthermore, the rate of transfer of acetaldehyde either into or out of the embryonic compartment during the early postimplantation period is not known since acetaldehyde has not been detected in fetal tissues (Kesaniemi and Sippel 1975). This result was probably due to the oxidation of acetaldehyde in both the placenta and the fetal liver (Sjoblom et al. 1978). In other studies, alcohol ingestion during pregnancy has produced specific patterns of congenital abnormalities. For example, both heavy and moderate alcohol use during pregnancy by humans and animals has been associated with reduced birthweights. Also, ethanol has been found to be teratogenic and embryolethal in the rat (Tewari and Crain 1980; Varna and Persaud 1979). Thus, the mechanisms of ethanol and acetaldehyde-induced toxicity and teratogenesis require much investigation.

In the present report we describe properties of an in vitro protein synthesizing system obtained from the human placenta. Human placenta is rich in protein synthetic activity and is rich in many enzymatic activities for protein biosynthesis. Our data showed that the cell-free systems from human placenta were active in supporting protein synthesis under in vitro conditions. Under our conditions, we obtained significant incorporation of (^{14}C) leucine and (^{14}C) phenylalanine into protein for at least 1 hour. Data also showed significantly higher incorporation of leucine than phenylalanine (table 1, figure 1). The data demonstrate that placental enzymes can actively carry out in vitro the transfer of (^{14}C) phenylalanine from (^{14}C) phe-tRNA to the ribosomal bound peptide bond. This reaction was highly stimulated by the presence of poly(U), the synthetic mRNA coding specifically for phenylalanine. Furthermore, the reaction, as expected, did not require ATP and actively proceeded in the presence of soluble factors and GTP. Thus, in common with many other in vitro systems, the present system is deficient in endogeneous mRNA.

The present results show that while the addition of 100 mM ethanol had no significant effect on the incorporation of (^{14}C) leucine or (^{14}C) phenylalanine into protein at all time points, 10 mM acetaldehyde under these conditions significantly inhibited the incorporation by 30-50 percent (figure 4). With addition of poly(U), the inhibition was greater, even though poly(U) stimulated the incorporation of (^{14}C) phenylalanine into protein by more than 800 percent. This experiment thus differentiated the effects of ethanol from those of acetaldehyde on the placental ribosomal system. Although these results suggest much further investigation, the data indicate that placental protein synthesis is more sensitive to the presence of acetaldehyde than to ethanol.

To understand the mechanisms of the activity and to precisely delineate the specific steps of protein synthesis affected by acetaldehyde, we also measured the transfer of (^{14}C) phenylalanine from (^{14}C) phe-tRNA to ribosomal bound polypeptide chains (table 4). This experiment enabled us to bypass the aminoacylation steps and concentrate on the reaction sequence leading to the ribosomal dependent peptide bond formation. It should be pointed out that the concentrations of both ethanol (100 mM) and acetaldehyde (10 mM) are high in these present studies (relative to common human in vivo levels). However, for in vitro studies, higher concentrations often are required to produce effects. For example, the peptidyl transferase reaction was found to be increased by the in vitro presence of high concentrations of ethanol (Monro and Marcker 1967; Monro 1969). Results of the experiment showed about 5 percent of the radioactivity is transferred and converted into polypeptides when the only source of message was endogenous ribosomal bound mRNA. The addition of poly(U) significantly stimulated the conversion to 25 percent. Ethanol had no effect on this process at 100 mM concentration but was inhibited slightly at 500 mM concentration (table 5). The lack of effects of 100 mM ethanol in the present system may again be related to the exposure time of the placenta to this chemical. Thus, the chronic or long-term exposure of the placenta to even low doses of ethanol may have different effects on the ribosomal properties than were seen for the in vitro effects.

Using (^{14}C) phe-tRNA, acetaldehyde was without effect or was even mildly stimulating at both 1 and 10 mM concentrations. However, at 100 mM concentration, acetaldehyde was extremely inhibitory, in both the presence and the absence of poly(U). Thus, the results indicate that at high concentrations, acetaldehyde interferes with the binding of messenger RNA to the ribosomal subunit or alters the peptide chain elongation process. Since the 60S ribosomal subunit catalyzes the peptidyl transferase reaction, acetaldehyde may have an effect on this process. However, it would appear that at the low concentrations present in vivo acetaldehyde may not appreciably affect the placental ribosomal protein synthetic activity, at least not acutely, although its long-term presence may have accumulative effects. Since 10 mM acetaldehyde was inhibitory when (^{14}C) phenylalanine was the precursor, but not when (^{14}C) phe-tRNA was used, acetaldehyde appeared to interfere with the aminoacylation steps of protein synthesis. We are further investigating these issues.

The small decrease due to ethanol additions shown in table 5, where we calculated the percentage of incubated CPM actually bound up in polypeptides, could result from defective attachment of poly(U) to ribosomes, which may have been functionally altered. On the other hand, if data were calculated using each endogenous mRNA condition as its own standard, compared to the values when poly(U) is added, other effects of ethanol and acetaldehyde are observed. This shows greater poly(U) dependent stimulation of (^{14}C) phenylalanine incorporation with the addition of either ethanol or acetaldehyde than in the standard conditions, thereby suggesting that some adverse effects of these drugs are exerted on the endogenous mRNA making the system deficient in available mRNA.

Our data point out that acetaldehyde rather than ethanol may be responsible for the placental toxicity. Since placental tissue is responsible for the secretion of several hormones and other proteins, interference or alterations of the protein synthetic properties will have devastating consequences, for both the embryo and the mother. In this regard it is important to note that ethanol consumption by pregnant rats throughout the entire gestation period or during specific trimesters of pregnancy causes a reduction of the in vitro brain protein synthesis in the fetal and neonatal brain (Tewari and Crain 1980; Tewari et al. 1980). Obviously, more experiments are required to examine these issues, particularly the effects of long-term ethanol exposure on placental protein synthesis.

Although our data do not deal with long-term exposure to heavy alcohol ingestion, the present in vitro condition could be compared to mild use or heavy one-time drinking by females during pregnancy.

Since the CNS is specifically vulnerable during each trimester of pregnancy, the implication of these findings is far reaching and may have great implications for pregnant women. Experiments are in progress to examine in detail the ethanol-acetaldehyde interaction with the protein biosynthesis of the placenta.

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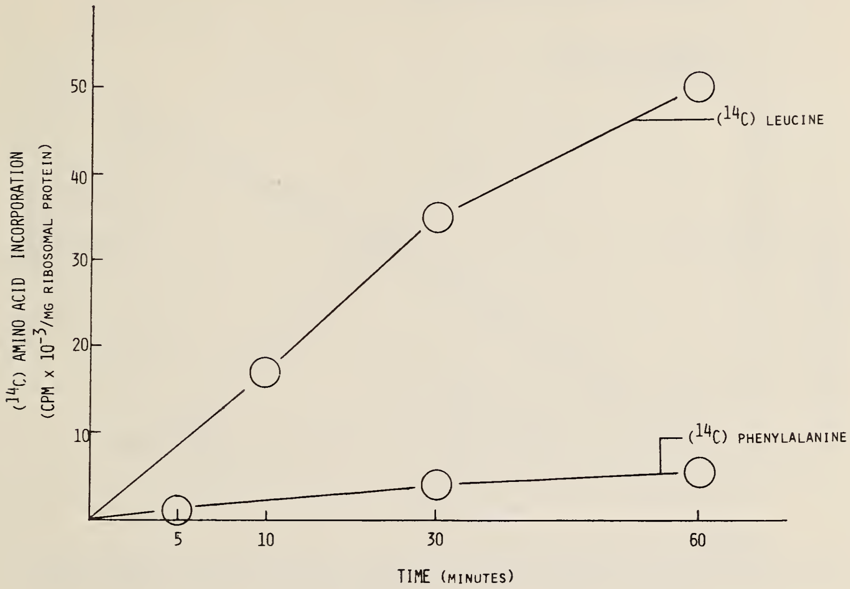
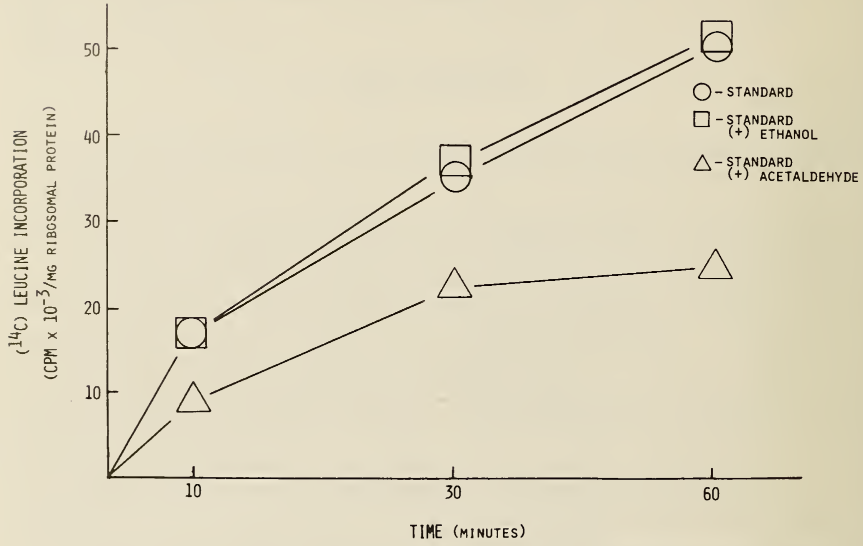
Figure 1. The Time Dependent in vitro Incorporation of (^{14}C) Leucine and (^{14}C) Phenylalanine into Protein



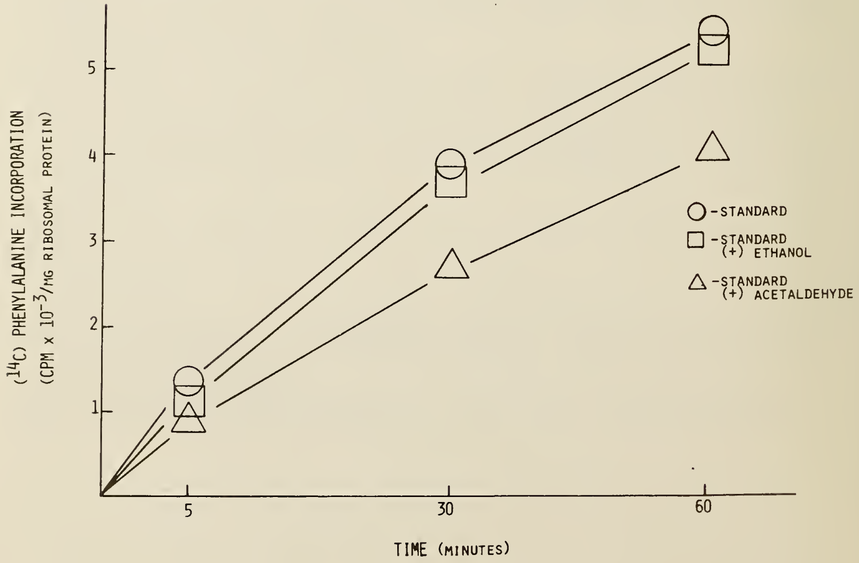
Experimental conditions are similar to those described in table 2 and the text.

Figure 2. **The Time Curve Incubation of Placental Ribosomes and pH5 Enzymes Fractions in the Presence and Absence of Ethanol and Acetaldehyde**

a. Ethanol Effects

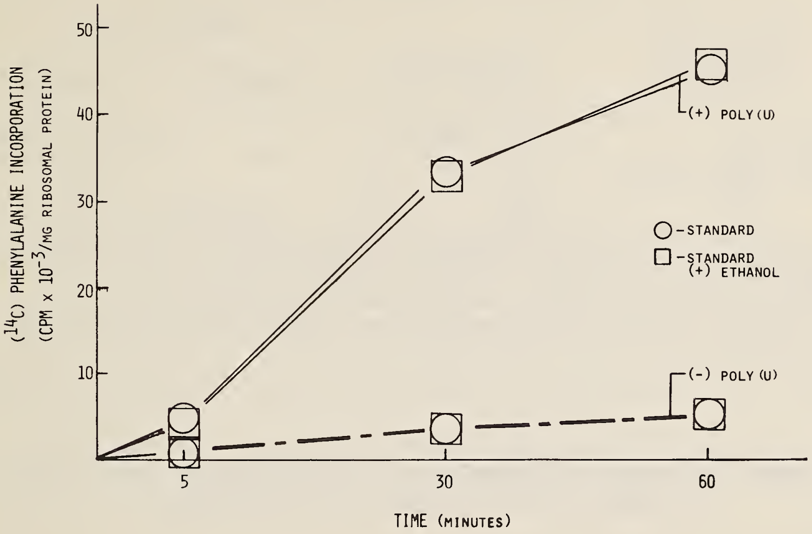


b. Acetaldehyde Effects



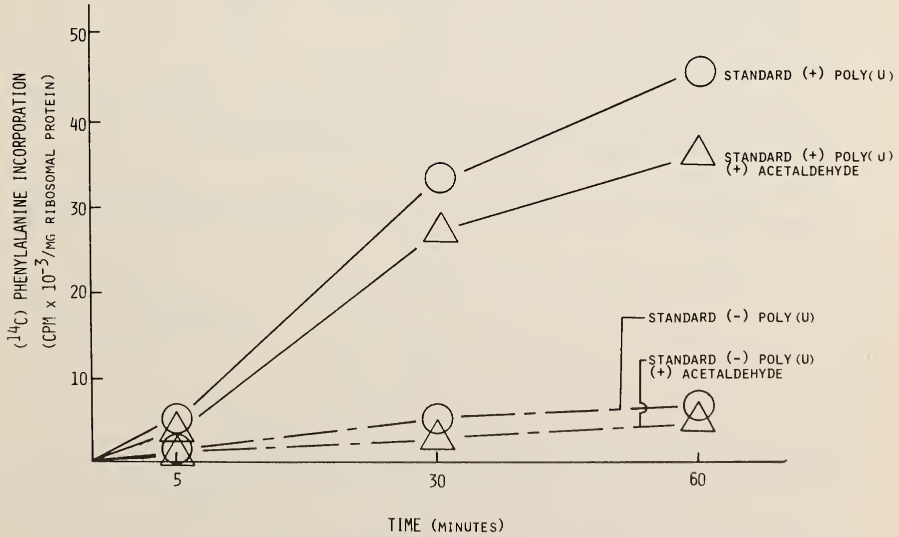
The incubation conditions are similar to those described in table 2.

Figure 3. The Poly(U) Dependent Protein Synthesis in the Presence and Absence of Ethanol



The incubation conditions are the same as previously described except poly(U) (when present) was added at concentrations of 100 μg/ml incubation medium.

Figure 4. The Poly(U) Dependent Protein Synthesis: Effects of Acetaldehyde



Experimental conditions were similar to those described in figure 3.

Table 1. The Biological Activity and Protein Content of Placental Subcellular Fractions

Subcellular Fractions	Protein Content mg/g Placenta	Biological Activity*	
		Leucine	Phenylalanine
Ribosomes	0.54	804	72
pH5 enzymes fraction	1.55		

Protein content was determined by methods as described in the text. The biological activity was measured by incubating the ribosomal and pH5 enzyme systems for 60 min at 37°C according to procedures described in the text. All assays were done in triplicates with variations rarely exceeding 2 percent of standard activity.

*Biological activity was expressed as $\mu\mu$moles amino acid incorporated/mg ribosomal protein.

Table 2. Effects of Ethanol and Acetaldehyde on the in vitro Incorporation of (^{14}C) Amino Acid into Protein

Precursor Amino Acid	Percent Standard Activity
(^{14}C) leucine	100*
+ 100 mM ethanol	100
+ 10 mM acetaldehyde	45
(^{14}C) phenylalanine	100**
+ 100 mM ethanol	93
+ 10 mM acetaldehyde	70

Incubation period was for 30 min at 37°C. Other incubation conditions were similar to those in table 1. All determinations were carried out in triplicates.

* 100 percent standard activity = 500 $\mu\mu$moles of leucine incorporated/mg ribosomal protein.

** 100 percent standard activity = 62 $\mu\mu$moles of phenylalanine incorporated/mg ribosomal protein.

Table 3. Effects of Ethanol and Acetaldehyde on Peptide-bond Conversion Using (^{14}C) phe-tRNA as the Precursor

Experimental Conditions	Biological Activity *		Percent Increase Polyphenylalanine Synthesized
Standard conditions	−poly (U)	+poly (U)	
No additions	3448	16502	479
+ 100 mM ethanol	3602	17236	479
+ 10 mM acetaldehyde	3480	17290	497

The incubation medium consisted of 1 ml of medium M containing (^{14}C) phenylalanyl-tRNA (13,620 cpm), 0.1 mM GTP, 0.2 mg ribosomes and 0.6 mg pH5 enzymes fraction. Incubation time was 30 minutes at 37°C. The incorporated radio activity was measured by determining (^{14}C) phenylalanine incorporation into hot TCA insoluble residue according to procedures described in the text. The (^{14}C) activities of phe-tRNA when determined in the incubation medium in the absence of ribosomes was soluble in hot TCA.

* Biological activity was expressed as (^{14}C) phenylalanine CPM incorporated/mg ribosomal protein. Poly(U) when present was added at the concentration of 100 μg/ml incubation medium. All assays were done in triplicate with variations rarely exceeding 5 percent of standard activity.

Table 4. The Conversion of (^{14}C) phe-tRNA to (^{14}C) Polyphenylalanine by Placental Ribosomes

Experimental Group	Total Recovered CPM in Hot TCA Insoluble Residue	Percent Radioactivity Transferred into Ribosome Bound Polypeptide Chain *
Standard Conditions		
− poly (U)	697	5
+ poly (U)	3301	24
Standard Conditions + ethanol (100 mM)		
− poly (U)	730	5
+ poly (U)	3468	25
Standard Conditions + acetaldehyde (10 mM)		
− poly (U)	879	6
+ poly (U)	3492	26

(^{14}C) phe-tRNA (13,620 cpm), poly (U), ribosomes and GTP in medium M were incubated for 30 minutes at 37°C as described in table 3. Poly (U) when present, was added at a concentration of 100 μg/ml incubation medium. Radioactivity was measured by hot TCA extraction.

* The recovered cpm were divided by the incubated cpm (13,620) and multiplied by 100.

Table 5. Effects of Varying Concentrations of Ethanol and Acetaldehyde on the Ribosomal Dependent Transfer of Radioactivity From (^{14}C) Phenylalanyl-tRNA to (^{14}C) Polypeptide

Experimental Conditions		Percent Transfer of Radioactivity *	
		− Poly (U)	+ Poly (U)
Standard Conditions		5.1	24.2
+ ethanol	100 mM	5.3	25.3
	500 mM	4.2	25.3
+ acetaldehyde	1 mM	5.0	25.0
	10 mM	5.1	25.4
	100 mM	0.45	2.3

* The incubations contained 13,600 cpm in (^{14}C) phenylalanyl-tRNA. The cpm obtained after incubation, hot TCA precipitation and collection of protein residue on millipore filters were divided by 13,600 and multiplied by 100 to get the above percentages. The incubation conditions are as described previously.

Table 6. Summary: Comparison of the Precursor Dependent In Vitro Effects of Ethanol and Acetaldehyde on Protein Synthesis

Precursor Amino Acid	Percent Standard Activity	
	+ 100 mM Ethanol	+ 10 mM Acetaldehyde
(^{14}C) leucine	100	45
(^{14}C) phenylalanine	93	70
(^{14}C) phe-tRNA	105	104

Experimental conditions were as described previously in table 1 and table 4. Poly(U) was not present under these conditions.

Alcoholic Pancreatitis

Henri Sarles and Rene Laugier

Introduction

Alcohol consumption is correlated with the frequency of both acute and chronic pancreatitis (Howard and Jordan 1960; Sarles et al. 1965, 1979). It is probable that acute alcoholic pancreatitis: (a) is observed in chronic drinkers only after paroxysmal increase of consumption (Kager et al. 1972); and (b) develops in pancreas presenting with more or less limited chronic lesions (Sarles et al. 1976; Strum and Spiro 1971). Therefore, alcoholic acute pancreatitis is probably a complication of incipient forms of chronic pancreatitis. Nevertheless severe acute episodes of chronic pancreatitis are more frequently observed in countries where alcohol consumption is paroxysmal on weekends (Kager et al. 1972; Marks et al. 1976) than in countries where the consumption is fairly regular, as in France (Sarles et al. 1965), Brazil (Mott et al. 1975), and Italy (Gullo et al. 1977). In this study alcoholic pancreatitis will be considered a chronic disease frequently associated with acute exacerbations.

Alcohol Metabolism by the Exocrine Pancreas

As the content of the exocrine pancreas in alcohol dehydrogenase and MEOS (the enzyme systems responsible for alcohol oxidation in the liver) is nil or low (Clemente et al. 1977; Schmidt and Schmidt 1960), it has been assumed that the pancreas does not metabolize ethanol. But Estival, Clemente, and Ribet (1980) found that the pancreas metabolized ethanol both in vitro and in vivo as much as a similar weight of liver. Three enzymes not yet described in the pancreas were found to be involved in alcohol oxidation: The first is a microsomal enzyme, producing an ethanol dependent NAD reduction. This activity was not found in microsomes of liver, kidney, fat, heart, and spleen. The second is another microsomal enzyme, the ethanol acyltransferase, responsible for the esterification of fatty acid with ethanol; the third corresponds to some aldehyde dehydrogenase activity. These findings need to be confirmed.

Nevertheless, ethanol at concentrations which can be reached in the

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NOTE: Figures appear at end of paper.

blood after oral administration of highly toxic doses has neither immediate toxic effect nor physiological action on the exocrine perfused pancreas of the dog (Fitzpatrick et al. 1975) nor of the rat (Clemente et al. 1977). Contradictory results were obtained only with higher alcohol concentrations impossible to obtain in the blood (Steer et al. 1979).

Pathological Study of Alcoholic Pancreatitis

The lesions of alcohol-induced pancreatitis have been studied by two different methods. Two observers conducted "blind examinations" of histological slides of pancreases from normal people and from patients presenting different types of pancreatitis (including alcoholic chronic pancreatitis). This study was done thanks to many previously defined features. When a disagreement was observed between the two observers, a third one was consulted and the opinion of the majority was kept. They compared pathological data between themselves and with physiological and nutritional data collected for each patient. This study was assisted by a computer program (Payan et al. 1972).

Serial slices of pancreases from normal people and from those with alcoholic pancreatitis were done from the wirsung duct to the most peripheral acini (generally 800 to 1,000 7.5 micrometre slices). Each slice was countertraced and a special reconstruction of the ducts done (Nakamura et al. 1972) (figure 1A and B). The conclusions of these two types of studies were consistent with each other: The first visible lesion at the optical microscope level in alcoholic chronic pancreatitis is the precipitation in the ducts of protein plugs. This has been recently confirmed (Sahel and Sarles 1979); such protein plugs were collected in the pure pancreatic juice of both alcoholic "controls" and even more frequently of patients presenting with alcoholic chronic calcified pancreatitis (Sahel and Sarles 1979). All transitions were observed between the protein films which can be found in normal juice and the protein plugs. At this stage neither fibrosis nor inflammatory infiltration nor destruction of cells was observed. Therefore, the first lesion of alcoholic pancreatitis to be found at the optical microscope level is the formation of protein precipitates in initially entirely normal ducts. At this stage a morphometric ultrastructural study of the exocrine pancreas has shown that acinar and duct cells were normal, acinar cells presenting only signs of hyperfunction (increase of cell and nucleus size, in endoplasmic reticulum length, of condensing vacuoles number and decrease of mature zymogen granules number) (Tasso et al. 1973).

Protein precipitates which may later calcify to stones are apparently responsible for atrophy and disappearance of duct epithelium and for multiple strictures of pancreatic ducts of all sizes, themselves secondary to lesions of the duct epithelium (cysts, retention-pseudocysts, atrophy of secretory tissue, acute lesions: edema, hemorrhagic necrosis, fatty necrosis, and necrotic pseudocysts) (Nakamura et al. 1972). Chronic pancreatitis presenting with this type of lesion can be easily separated from chronic pancreatitis upstream to a stricture of the main duct (Payan et al. 1972) by the association and the particular frequency of these different features: Presence of calcified (stones) or not calcified protein precipitates in the ducts; important alteration of duct epithelium; lobular, spotty distribution of the lesion. At the beginning, one lobule presents with lesions in the middle of normal exocrine tissue; later, the whole exocrine tissue is partly or totally destroyed (figure 2). This distribution of the lesions seems to be explained by the fact that protein precipitates and stones are randomly found in different ducts.

It has been shown (Nakamura et al. 1972) that in the territories which present with advanced lesions and destruction of cells, there was an infiltration of mononuclear cells. In certain cases, these cells are grouped into cell strains and clouds that show the pattern of the destroyed ducts and lobules. Lymphocytes are also abundant in the perinervous lymphatic trunks. It is therefore possible that lymphocytes and immune mechanisms play a role in the destruction of these cells (Dani et al. 1974). In conclusion, for this type of disease we propose the name of calcifying chronic pancreatitis (CCP). CCP is not specific of alcoholic pancreatitis but is also found in hypercalcemia (hyperparathyroidism), tropical pancreatitis, hereditary pancreatitis, some rare cases of adult cystic fibrosis, and in the so-called idiopathic pancreatitis (Sarles and Howat 1979). Therefore, as the lesions are similar for these different etiologies, some common mechanisms must be found.

Biochemical Study of Pancreatic Juice in Alcoholics

As pancreatic protein plugs (identical to protein precipitates found in pancreatic juice by ERCP) are the first visible lesions of CCP, the biochemical study of the alcohol-induced modifications of pancreatic juice, of the intraductal protein precipitates, and of their last stage, the pancreatic stones, should enlighten the alcohol-induced pancreatic pathogenesis (Sarles et al. 1980).

Alcohol-Induced Modification of Pancreatic Juice

An increased concentration of protein (Sahel and Sarles 1979) has been found in the pure pancreatic juice of alcoholic "controls" (with apparently normal pancreas) and of alcoholic patients with CCP (protein concentration was even higher for CCP patients than for alcoholic controls). Similarly, Renner, Rinderknecht, and Douglas (1978) found a very high concentration of proteins in the pure pancreatic juice following each alcoholic abuse. The same authors confirmed the increased protein secretion of alcoholic patients but without statistical study matching these patients with nonalcoholic controls. Renner's patients have a higher protein concentration than our patients, who were studied in the weeks following the cessation of alcohol consumption; the California patients were studied immediately after alcohol consumption. Therefore, it is possible that chronic alcohol consumption is responsible for an increased concentration (and probably output) of protein, which is at its maximum immediately after alcohol consumption but persists for at least some weeks. As the differences between alcoholics and nonalcoholics were similarly observed in basal secretion after a meal and after injection of exogenous hormones (secretin, CCK) (Palasciano and Sarles 1977; Sahel and Sarles 1979; Sarles et al. 1980), it is probable that these differences are due to modifications of basal secretion. Beside the fact that protein precipitates are more frequently observed in pure pancreatic juice of alcoholics (either presenting or not with CCP) than of nonalcoholic controls, Renner et al. (1978) found in the first group more frequently an activation of trypsinogen that could play a role in the formation of acute lesions but also be a mere consequence of protein hyperconcentration. Similarly, in dogs submitted to 1 year alcohol consumption (2 g/kg/day), the basal concentration of protein is increased (Noël-Jorand et al. 1980).

In contrast to protein, bicarbonate concentration is decreased in chronic alcoholics (Sahel and Sarles 1979; Sarles et al. 1980). As volume is not increased, this corresponds to a modification of bicarbonate secretion. For protein this effect is mostly observed in basal secretion. Similar results have been found in the dog (Noël-Jorand et al. 1980) where pure pancreatic juice pH is also decreased in alcoholics. These modifications of the ionic environment of proteins could play a role in their precipitation. Although the pancreatic lesions are thought to be mostly a consequence of pancreatic secretion changes, it is not ruled out that some among the ionic modifications could also be secondary to pancreatic lesions (atrophy of duct epithelium and transudation of interstitial fluid) and directly related to alcohol consumption.

Calcium (Gullo et al. 1974; Vêrine et al. 1977) and serum protein (including serum albumin) concentration (Clemente et al. 1971) are increased in pancreatic juice of CCP patients. Serum albumin concentration in pure pancreatic juice gives an idea of the evolution of pancreatic lesions (Multigner et al. 1980). Similarly, the increased concentration of lysosomal enzymes (Rinderknecht, Renner, and Koyama 1979) could be a nonspecific consequence of cell destruction as it is also increased in cancer (Rinderknecht and Renner 1980).

According to Lohse et al. (1980), citrate secretion in duodenal juice is decreased in alcoholic compared to nonalcoholic man. This difference is observed but is not significant in the pure pancreatic juice of our patients (Sarles et al. 1980). Citrate, which can solubilize pancreatic stones and plugs, is normally secreted and could play a part in the solubility of calcium carbonate and serum proteins (De Caro et al. 1979; Lohse et al. 1980).

Finally, some suspected modifications are probably due to technical artefacts. (1) The decrease of trypsin inhibitor (Rinderknecht, Renner, and Carmack 1979) cannot be estimated by biochemical methods because trypsin is partly activated. (2) The modification of the ratio trypsin I to trypsin II (Rinderknecht, Renner, and Carmack 1979) has not been confirmed in our laboratory by more sophisticated methods. It could be due to the different stability of the two isoenzymes of trypsinogen (Guy et al. 1978). (3) The increased secretion of water and bicarbonate in duodenal juice of alcoholic man (Dreiling et al. 1973) is due to an increased biliary secretion in case of alcoholic hepatic lesions (Bode et al. 1971) and not to the increase in pancreatic secretion. On the contrary, in the dog at the end of 2 years of alcohol consumption (2 g/kg/day) there is an increased response of water and bicarbonate to secretin and to CCK (Sarles et al. 1977).

In conclusion, many alcohol-induced modifications of the composition of pancreatic juice could play a role in the precipitation of proteins and formation of protein plugs and stones. Some arguments focus even more the pathological role of an increased protein to bicarbonate ratio in pancreatic juice. As shown above, hypercalcemia and adult cystic fibrosis are responsible for lesions similar to those induced by alcohol. Chronic hypercalcemia in the dog induces persistent increase in basal protein secretion (bicarbonate secretion being not modified), and the formation of protein precipitates in the juice (Noël-Jorand et al. 1980). Acute and calcifying chronic pancreatitis, which are similar to alcoholic pancreatitis, are observed in hypercalcemic patients (Dubost et al. 1979; Mixter et al. 1962). During the early stages of cystic fibrosis, protein secretion is normal, water and bicarbonate secretion being decreased (Hadorn et al. 1968). In cystic fibrosis, acute pancreatitis (Schwachman et al. 1975) and chronic calcifying pancreatitis (Di Sant'Agnese and Leport 1961) are observed as well.

Composition of Protein Precipitates

Protein precipitates collected at ERCP are made of two fractions (Sarles et al. 1980). One is an easily soluble fraction which contains the different normal secretory pancreatic enzymes including trypsin, which can be in the nonactivated form (trypsinogen): activation is not necessary for precipitation. The second fraction is solubilized by citrate and contains different proteins including a specific protein of pancreatic calculi, the "stone protein."

Composition of Pancreatic Calculi

Pancreatic stones from alcoholic chronic pancreatitis patients may be dissolved very slowly in isotonic saline and their solubility increased by citrate (Lohse et al. 1980). This allows biochemical study of the pancreatic stones; they are built up from calcium carbonate in the form of calcite (Periner et al. 1972) and from proteins. These proteins have been recently studied. A major protein is found in all stones (figure 3), corresponding to the stone protein. It has a molecular weight of 13,500 daltons and has no known enzymatic activity. It is present in the normal and pathological nonactivated and activated pancreatic juice (De Caro et al. 1979) (figure 4). It has a high concentration of lysine and aspartic and glutamic acid (Lohse et al. 1978), which explains its high affinity for calcium. It is sometimes alone pure in the stone, sometimes associated with other proteins probably representing pancreatic enzymes (unpublished data).

Mechanisms of Stone Formation

The most reasonable assumption to explain pancreatic stone formation is that ionic modifications of pancreatic juice are induced by chronic alcohol consumption as well as by hypercalcemia (increase of the protein to bicarbonate ratio). These modifications are responsible for the precipitation of all secretory proteins (including the stone protein) and calcium carbonate; most proteins are washed out but the stone protein remains, forming insoluble complexes with calcium carbonate. The role of a decreased citrate secretion should be confirmed, and whether the secretion of the specific stone protein is modified by chronic or acute alcohol consumption should be determined.

Mechanisms of the Modifications Induced by Chronic Alcohol Consumption

Mechanisms that can help the understanding of alcohol-induced pancreatic lithogenesis are studied mostly in men and dogs (Sarles 1976). The rat behaves differently from man and dog; intravenous ethanol, in particular, has no action on the basal secretion of nonalcoholic rats with diversion of pancreatic juice (Demol et al. 1980). On the contrary, the contact of ethanol with the duodenal mucosa induces a strong secretion of protein and bicarbonate (Demol et al. 1980). In addition, the action of ethanol on the rat pancreas is extremely variable: alcohol consumption increases the enzyme content of the rat pancreas when associated with a high fat, high protein diet but decreases it when associated with a low protein, low fat diet (Sarles and Figarella 1967). Prolonged alcohol consumption increases pancreatic protein secretion at the end of 4 to 8 months but decreases it at the end of 12 months. The hypersecretory state persists longer in some animals (Laugier and Sarles 1977). In pancreatic lobules drawn from alcohol-fed rats for 3 months, an increase in basal secretion of lipase, chymotrypsin, and trypsin has been observed (Bode et al. 1980). This explains why different authors have found that alcohol either increases or decreases pancreatic protein secretion. Moreover, alcohol consumption in the rat is responsible for an increased number of lesions which may be observed in nonalcoholic animals as well and resembles CCP (Sarles et al. 1971). Since the number of these lesions varies in apparently similar conditions (unpublished data) they could be due to lesions of the arteries. Nevertheless, in the series of animals where protein precipitates (sometimes calcified) have been found, pancreatic juice always presented with protein hyperconcentration (Sarles et al. 1971) and the transit time of newly synthetized proteins from the zymogen granules area to the acinar lumen was decreased (Lechêne de la Porte and Sarles 1974), indicating an increased secretion.

Hormonal changes could induce the earliest modification of human and canine pancreatic secretion (an increased concentration of protein in basal juice; see above).

—CCK release is not modified in chronic alcoholic dogs (Planche et al. 1977; Sarles et al. 1977).

—Gastrin release is increased in chronic alcoholic men (Treffot et al. 1980) and dogs (Treffot et al. 1975). This could play a role in dogs but probably not in man since gastrin has a very weak secretory action on the human pancreas.

—Secretin release is decreased in alcoholic dogs following the injection of HCl into the duodenum but the basal secretin release is not modified (Bretholz et al. 1978). In man the response of radioimmunologically estimated secretin to 100 ml vodka per os has been studied (Raptis et al. 1978): Secretin increases from a basal level of 20 pg/ml to 60-70 pg/ml and returns to normal levels in 50 min. This release is abolished by an infusion of 250 μg/hr somatostatin (Raptis et al. 1978). Conversely, the injection of 60 ml pure vodka into the duodenum does not modify secretion blood levels (Fahrenkrug and Schaffalitzky de Muckadell 1977). Therefore, potent stimulant of gastric secretion, the action of alcohol on secretin blood levels is probably exerted through the secretion of H^+ by the stomach.

—In dogs at the end of 3 months of alcohol feeding (2 g/kg/day) the sensitivity to secretin is not modified but the response to CCK is increased (Sarles et al. 1973). At the end of 2 years the response to CCK is not significantly modified. At the end of 2 years but not at the end of 1 year, bicarbonate concentration and output in response to high doses of secretin are increased compared to nonalcoholic dogs (Sarles et al. 1973). This is explained by hyperplasia of the ducts and is different from results obtained in chronic alcoholic men in whom bicarbonate output increase is not observed.

Therefore, the sensitivity of the human pancreas to hormones being apparently not modified, their role in the hypersecretion of proteins seems to be weak, unlike the role of the alcohol-induced release of acetylcholine; this is illustrated by the fact that in the pancreatic nerve endings acetylcholine esterase is decreased but acetylcholine transferase is increased (Celener et al. 1977; Perec et al. 1979).

Some indirect arguments focus on the possible role of an increased cholinergic tone induced by chronic alcohol consumption:

—In alcoholic chronic pancreatitis, the secretion of sodium and chloride by the sweat is increased (Sarles et al. 1965). (The role of cystic fibrosis and adrenal insufficiency was excluded.)

—An insecticide which blocks acetylcholine esterase is a cause of acute pancreatitis (Dressel et al. 1979).

—Repeated treatment with reserpine depletes catecholamine and induces a high protein, low bicarbonate secretion (Voirol et al. 1976) and is responsible for pancreatic lesions similar to chronic calcifying pancreatitis (Setser et al. 1979).

—In man as in dog, intravenous injections of nontoxic doses of ethanol (0.7 to 1.0 g/kg) have different actions if the subject of the experiment is alcoholic or nonalcoholic and accustomed to the regular consumption of alcohol.

Intravenous ethanol in normal subject decreases pancreatic secretion induced by secretin, CCK, or gastrin (figure 5) (Capitaine et al. 1971; Mott et al. 1972; Planche et al. in preparation). This is true for control dogs as well (Tiscornia et al. 1972). This action is suppressed by anticholinergic drugs both in man (Meullenet et al. 1979) and in dogs (Tiscornia et al. 1973, 1975).

Recently we have shown that alcohol-induced inhibition of pancreatic secretion in man was correlated (figure 6) with an alcohol-induced release of pancreatic polypeptide (PP) at concentrations sufficient to decrease pancreatic secretion (Staub et al. submitted). The correlation with somatostatine-like products and VIP was not observed. As PP release is transmitted by the vagus and muscarinic receptors, it could explain at least some part of the alcohol-induced inhibition of pancreatic secretion in nonalcoholic dogs and men.

In chronic alcoholic man intravenous ethanol has no more significant action on pancreatic secretion (Planche et al. in preparation). This could be due to the fact that in chronic alcoholic pancreatitis it has been found that PP release is abolished (Valenzuela et al. 1979). In chronic alcoholic dogs, the inhibitory effect of intravenous alcohol is not only suppressed but also replaced by an excitatory action upon volume, bicarbonate, and protein outputs (figure 7). Atropine is still able to abolish this excitatory effect of acute alcohol (Tiscornia, Palasciano, and Sarles 1975), but pentholinium (Tiscornia et al. 1974) and vagotomy (unpublished data) are not. Therefore, alcohol probably acts at the level of intrapancreatic ganglion cells of the vagus nerve. At the end of 3 years the excitatory effects of intravenous ethanol in the dog disappear, alcohol being unable to modify pancreatic secretion (unpublished data).

Finally, the assumption of an increased intrapancreatic cholinergic tone is supported by histochemical studies realized in our laboratory which have shown that acetylcholine-esterase is decreased and choline-acetyltransferase increased in the nerve endings of chronic alcoholic (CA) pancreases compared to nonalcoholic controls (unpublished data). A series of arc reflexes probably controls the intrapancreatic cholinergic tone. One of these mechanisms has been shown by Thambugala and Baron (1971): Under prolonged secretin infusion a topical anaesthesia of duodenal mucosa at the level of pancreatic papilla decreases pancreatic secretion. This effect has been confirmed and it has been shown that it was reduced in CA dogs (Tiscornia et al. 1976). Conversely, when CCK is added to the secretin infusion, the mucosal anaesthesia increases pancreatic secretion of protein. This effect is increased in CA dogs.

During the course of chronic ethanol consumption (Tiscornia et al. 1977*b*) or when alcohol is added to a normal meal (Tiscornia et al. 1977*a*), the response of the dog's pancreas to the meal is characterized by a decrease of bicarbonate secretion. This inhibitory action of alcohol on bicarbonate secretion could be the near consequence of lesions induced by hyperconcentrated juice proteins but may well also be in relation with acetaldehyde formation. In the dog (Andersen et al. in press) it is not very probable that the concentration of acetaldehyde that can be reached in the blood during alcohol consumption could play a role, but if alcohol is really metabolized by the pancreas (Estival et al. 1980), local concentration of acetaldehyde could be sufficient to interfere with carbonic acid anhydrase and acetic acid could replace bicarbonate in pancreatic secretion.

Statistical Relation Between Alcohol Consumption and Risk of Chronic Pancreatitis

The statistical relationship between alcohol consumption and risk of pancreatitis has been studied by Durbec et al. (1978, 1980). Patients were matched with normal controls randomly drawn from the population and with functional patients. The mean daily consumption of alcohol was divided into groups of 20 g each (0; 1-20; 21-30; etc.), the first group being reserved for nondrinking patients.

There is a linear relationship between the logarithm of the relative risk of developing alcoholic pancreatitis (risk related to the class 0) and the mean daily alcohol consumption. Therefore, there is no statistical threshold of alcohol toxicity but rather a continuous spectrum of individual thresholds from the lowest tolerances to the highest ones (figure 8). The slope of the curve is even greater between the two first classes. Thus it is difficult to know what to consider idiopathic pancreatitis.

The duration of alcohol consumption is also a factor of risk: For the same duration of alcohol consumption the risk increases with the increase of alcohol intake.

The type of beverage, wine, whisky, beer, etc., has no role. If carbohydrate does not play a role, protein and fat modify the risk (figure 9). For alcohol there is a linear correlation between the protein intake and the logarithm of the relative risk, but the relative influence of protein is much lower than the weight of alcohol. The relation with fat is quadratic: The risk is lowest with the mean consumption (80-100 g/day), higher with low fat diet, and even higher with high fat diets. The noxious effect of fat and protein has been confirmed by Gullo et al. in Italy (1977), Goebell et al. in Germany (1980), and Mott in Brazil (1975). The effects of fat, alcohol, and protein are additive. The data from Pitchumoni (1980), who found in Veterans Administration patients a very high alcohol intake associated with low protein, low fat diet, could be explained by the fact that these patients were probably questioned at the end of the disease when they had modified their diets spontaneously, whereas our patients were recent patients who did not modify their diets. In addition, Pitchumoni did not use a control population.

The additive effect of fat, protein, and alcohol partly explains why only a certain number of alcoholics develop chronic pancreatitis. Besides these acquired nutritional factors, it is possible that congenital factors may play a role. The number of families with two cases of alcoholic pancreatitis or two cases of chronic pancreatitis, one alcoholic and the other not, is probably higher than by chance (Sarles et al. 1966). Marks and Bank (1976) described the abnormal number of blood group "O," and some other congenital markers in patients with chronic calcifying pancreatitis. Conversely, the different repartitions of HLa antigen (Dani et al. 1978) seem to be an artefact.

Some authors have found in the pancreatic juice of patients with alcoholic, hereditary, and idiopathic pancreatitis an abnormally high concentration of a particular protein, the lactoferrin (Colomb et al. 1976; Estevenon et al. 1975; Fedail and Harvey 1970). Lactoferrin is increased neither in acute pancreatitis nor in pancreatic cancer (Multigner et al. 1980). According to our most recent data, 4 to 6 percent of the normal population has an increased concentration of lactoferrin in the pancreatic juice. Lactoferrin concentration is a function neither of alcohol consumption nor of the degree of lesions. We are testing the assumption that it is a genetic marker for an acquired or a congenital sensitivity of the pancreas to ethanol.

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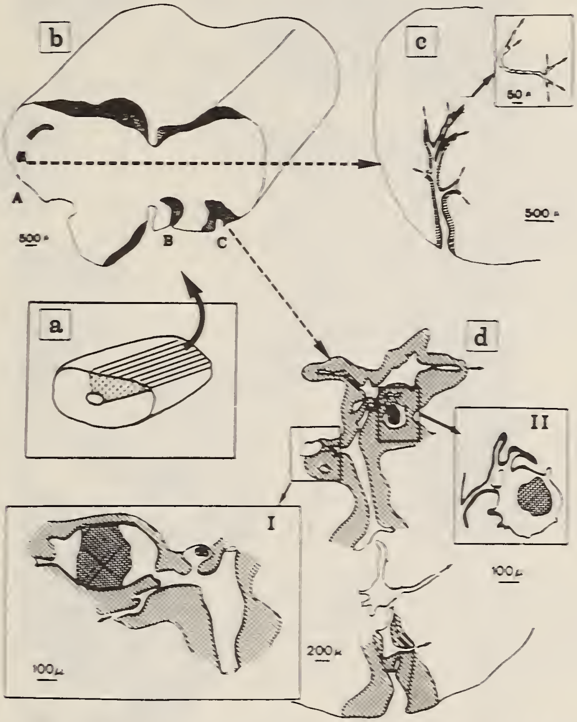
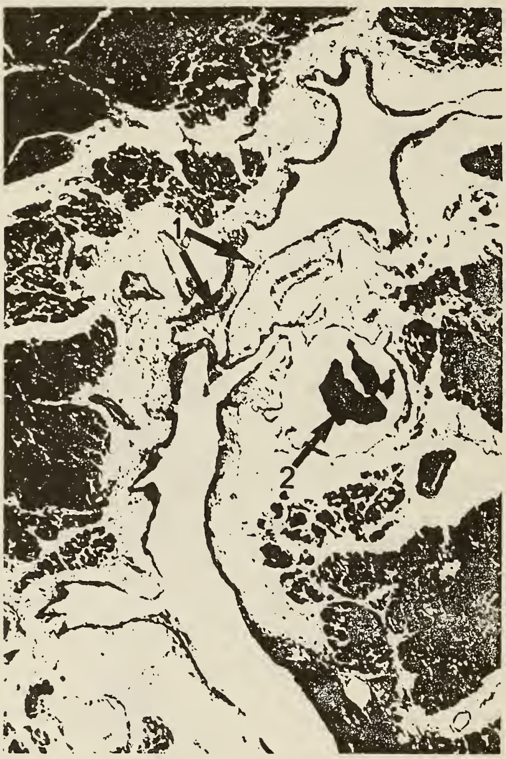
Figure 1a. Projection of a Reconstructed Duct in a Plane Parallel to Its Axis, Yet at Right Angle with Wirsung



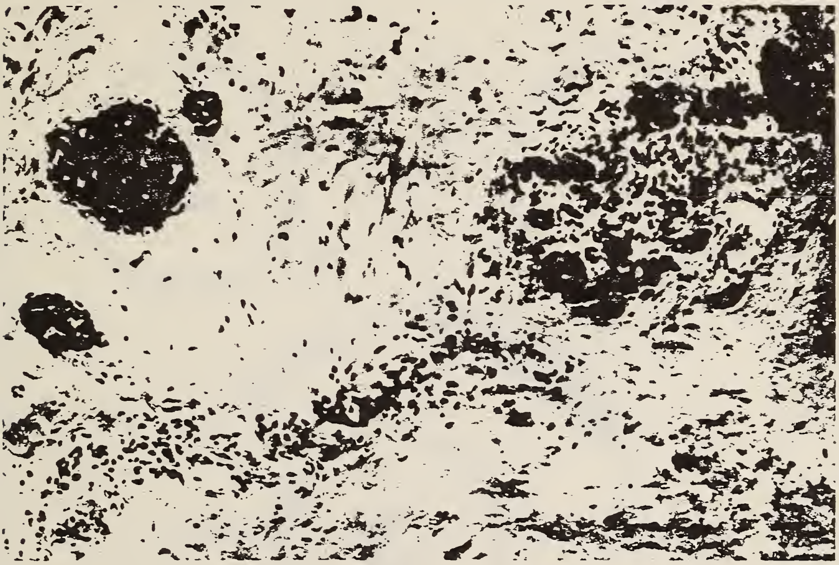
The specimen (b) was obtained by unroofing the pancreas (a) of a patient with early calcifying pancreatitis. Cross-hatched areas represent fibrosis; the squared areas, protein plugs within ducts; stippled areas, lobules normal features of which are partially or completely preserved. When the wall of the duct is indicated by a dotted line, the drawings in solid line which cross their path represent the contours of the duct in the section at this level. The details of secondary ducts at position A are shown in drawing c and of duct at position C in drawing d. Both ducts have been followed up as far as their point of origin in lobules; duct A (a) communicates with normal lobules. Two dilated ducts (I and II) containing protein plugs are shown at higher magnifications of duct C (d).

Figure 1b. Higher Magnification of Duct B



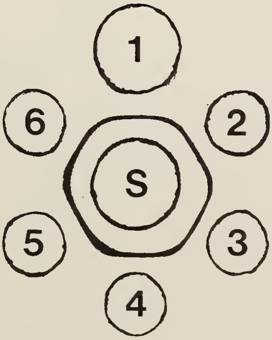
A dilated duct is seen to contain a dense protein plug (2). In (1) a protein film is seen which represents the limit of what may be found in normal ducts. From Nakamura, K.; Sarles, H.; and Payan, H. Three dimensional reconstruction of the pancreatic ducts in chronic pancreatitis. *Gastroenterology*, 62(5):944-945, 1972. Copyright 1972 by The Williams and Wilkins Co. (Baltimore).

Figure 2. Example of an Advanced Stage of Chronic Calcifying Pancreatitis



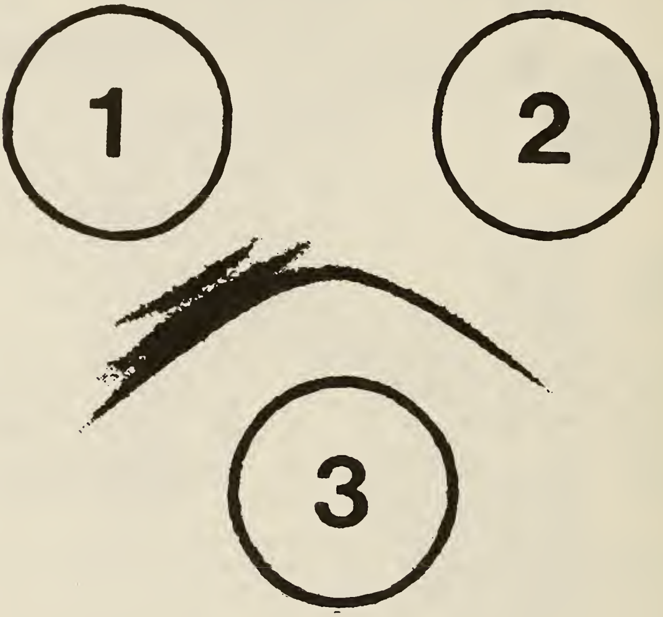
The only remains of an interlobular duct is a strand of mononuclear cells. The site of the lobule itself is shown by a collection of mononuclear cells and by groups of acinar cells and ducts, for the most part without a lumen. From Nakamura, K.; Sarles, H.; and Payan, H. Three dimensional reconstruction of the pancreatic ducts in chronic pancreatitis. *Gastroenterology*, 62(5):946, 1972. Copyright 1972 by The Williams and Wilkins Co. (Baltimore).

Figure 3. Ouchterlony Double Diffusion Between the Protein Isolated from Six Different Human Pancreatic Calculi (Outer Wells) and Antiserum to Human Pancreatic Juice (Center Well)



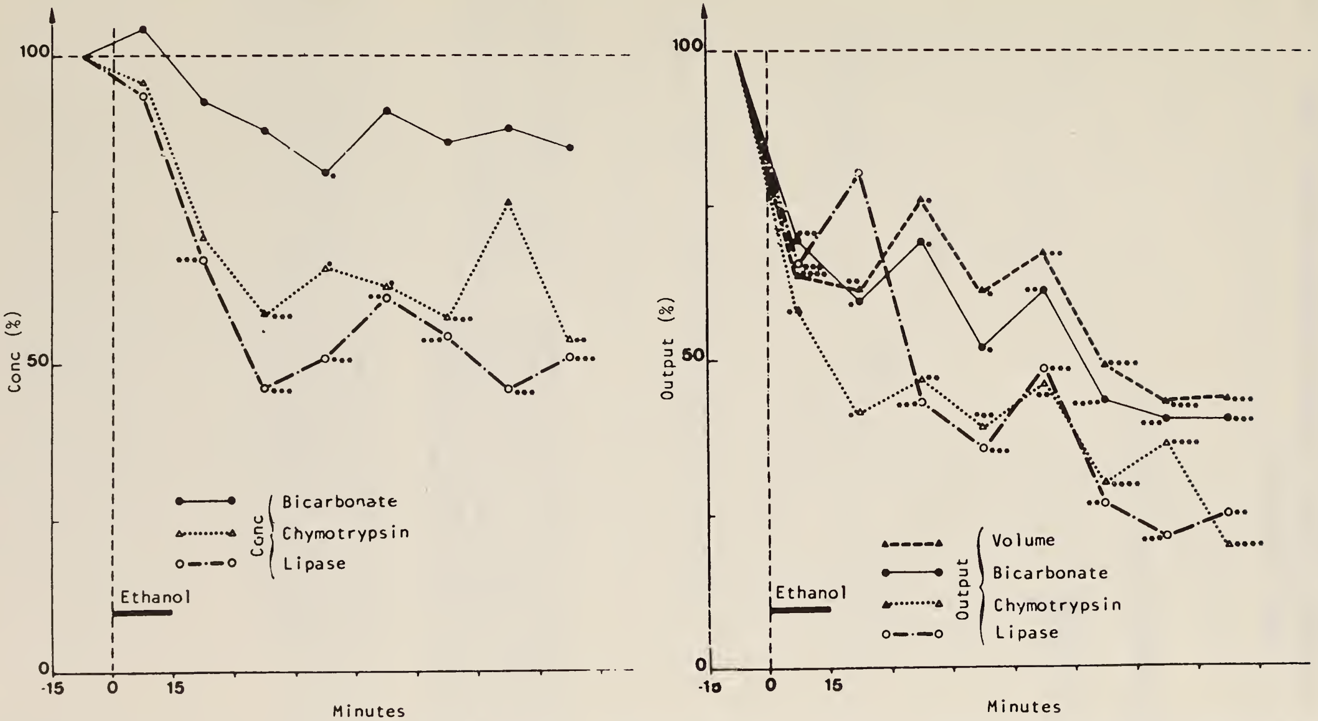
There is no immunological difference between the major protein extracted from these different calculi. Reprinted with permission from De Caro et al. in *Biochemical and Biophysical Research Communications*, 87(4):1178. Copyright 1979.

Figure 4. **Cross-Reactions Between Pancreatic Proteins of Low Molecular Weight and Protein Isolated from Pancreatic Stones**



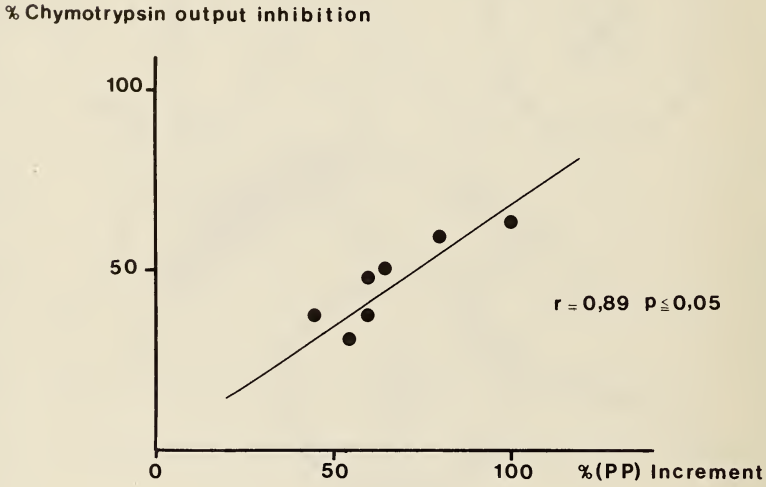
Ouchterlony double diffusion between human pancreatic protein of low molecular weight (1), protein isolated from pancreatic stones (2), and antiserum to human pancreatic proteins (3). This "protein stone" is present in fractions of low molecular weight obtained by gel filtration from normal human pancreatic juice. (From De Caro, A.; Lohse, J.; and Sarles, H. Characterization of a protein isolated from pancreatic calculi of men suffering from chronic calcifying pancreatitis. *Biochem Biophys Res Commun*, 87(4):1180, 1979. Copyright 1979 by Academic Press, Inc. [NY].)

Figure 5. Percentage Changes and Statistical Significance of the Reduction in Volume, Concentration, and Output of Bicarbonate, Lipase, and Chymotrypsin After Intravenous Ethanol Infusion



Ethanol infusion: (10 percent ethanol. 600 mg/kg for 15 min.). Hundred percent is the last sample collected before alcohol infusion (with a background infusion of 0.25 CHR.U/kg/h secretin + 1.0 CHR.U/kg/h CCK). In the margin are noted the different levels of significance between each value and the plateau level. (From Mott, C. de B.; Sarles, H.; Tiscornia, O.; and Gullo, L. Inhibitory action of alcohol on human exocrine pancreatic secretion. *Am J Dig Dis*, 17(10):907, 1972. Copyright 1972 by Plenum Publishing Corp. [NY].)

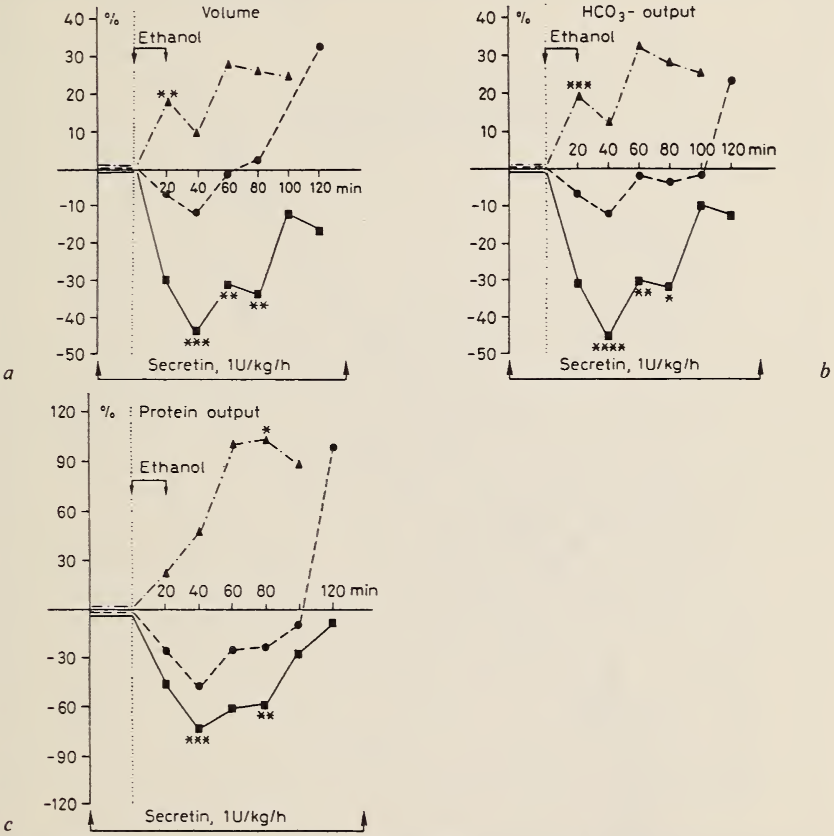
Figure 6. **Correlation Between Percentage Mean Inhibition of Chymotrypsin Output in Human Pancreatic Secretion and Percentage Mean Increase of Blood Levels of Pancreatic Polypeptide**



Correlation during the first 45 min. following IV ethanol infusion (600 mg/kg for 15 min.). Hundred percent is plateau values obtained with continued IV infusion of 0.25 CHR.U/kg/h secretin + 1.0 CHR.U/kg/h CCK. Reprinted with permission, from J.L. Staub et al., Relation between the alcohol-induced inhibition of pancreatic secretion and plasma levels of pancreatic polypeptide, somatostatine and vasoactive intestinal peptide, submitted to *Regulatory Peptides.*

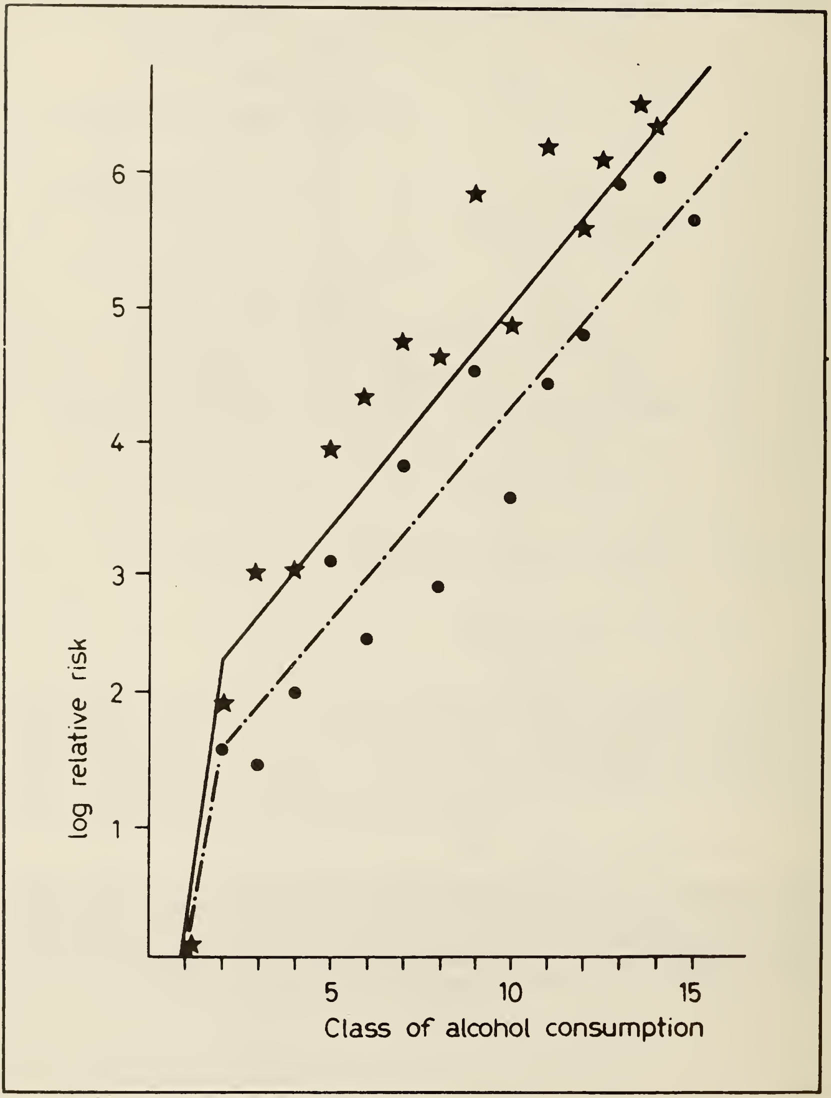
Figure 7. Percentage Changes and Statistical Significance of the Variations of Volume, Bicarbonate, and Protein Output, After Intravenous Alcohol in Chronic Alcoholic Dogs

Effects of Chronic Ethanol Administration on Canine Exocrine Pancreatic Secretion



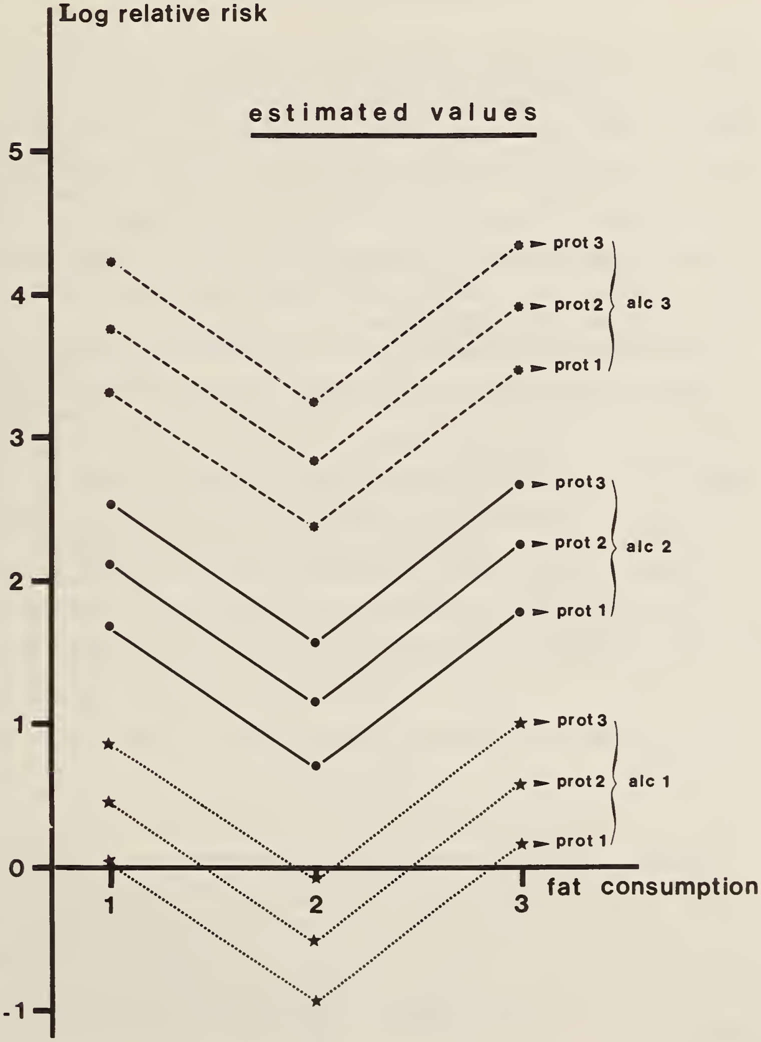
Hundred percent is the last sample collected under continuous IV infusion of 1.0 U/kg/h secretin. ■, nonalcoholic dogs; ● 3 months chronic alcoholic dogs; ▲ 12 months chronic alcoholic dogs. Significant differences with plateau values at $p < 0.05$, 0.02 and 0.005 levels are indicated by *, **, *** respectively. Reprinted with permission, O. Tiscornia et al., in *Digestion*, 11:175, 1974. Copyright 1974 by S. Karger (Basel).

Figure 8. **Graphic Representation of the Log-Relative Risk of Developing a CCP as a Function of Alcohol Consumption Class**



International survey; o observed, —•— estimated; Marseille survey: * observed, ____ estimated. From Durbec, J.P. and Sarles, H. Multicenter survey on the etiology of the pancreatic diseases. Relationship between the relative risk of developing chronic pancreatitis and alcohol, protein and lipid consumption. *Digestion* 18:337-350, 1978. Copyright 1978 by S. Karger (Basel).

Figure 9. Estimated Values in the International Survey of the Log-Relative Risk as a Function of the Fat Consumption Classes for the Different Classes of Alcohol and Protein Consumption



Reprinted with permission, from Durbec, J.P., and Sarles, H. Multicenter survey on the etiology of pancreatic diseases. Relationship between the relative risk of developing chronic pancreatitis and alcohol, protein and lipid consumption. *Digestion* 18:337-350, 1978. Copyright 1978 by S. Karger (Basel).

Discussion of Paper by Sarles and Laugier

David A. Dreiling

I have read with pleasure the many significant contributions made over the past 25 years by Professor Henri Sarles and his group, contributions which have done much to elucidate pancreatic pathophysiology. Henri and I have always had our little differences, but in time our views grow closer. We both agree that an early response to injury is hypersecretion. Henri believes that this results from the effects of increased vagal activity releasing CCK-PZ and sensitizing the acinar cells to the hormone—thence protein hypersecretion, proteinaceous plugs—the small duct hypothesis.

I believe the hypersecretion is due to hypertrophy and hyperplasia of the parenchyma and have found this response to follow many types of pancreatic injuries (see table 1), both experimental and clinical: (1) alloxan; (2) ethionine; and (3) ductal ligation. It can be seen in the asymptomatic alcoholic during the so-called latent period.

Histologic examination of pancreatic biopsies obtained in this group of alcoholics without clinical pancreatitis shows distinct cellular pathologies: (1) intracellular deposition of fat; (2) endoplasmic reticular hyperplasia; (3) mitochondrial distortions; and (4) proteinaceous precipitates in the endoplasmic reticulum. These changes suggest a toxic metabolic disturbance preceding and perhaps producing the plugs.

Henri, at least we both have discarded the simplistic mechanistic hypothesis.

Table 1. Post Secretin Data Pancreatic "Hypersecretion"

	#	Ave. 80′ Flow ml/kg	Ave. Max HCO_3 meq/L	Ave. 80′ HCO_3 Secretion meq
Alcoholism	28	4.9	95	30.6
Biliary Cirrhosis	44	4.4	108	32.4
Nonalcoholic Cirrhosis	39	4.5	106	36.5
Alcoholic Cirrhosis	95	6.2	94	43.2
Hemachromatosis	19	9.4	96	60.9

Comment on Paper by Sarles and Laugier

Romano C. Pirola

If I had only one comment on Professor Sarles's paper, it would be to congratulate him on proposing a theory that has the attraction of being applicable to most forms of pancreatitis, be they alcoholic or of other etiologies. Furthermore, he has presented us with a number of elegant experiments with findings compatible with his hypothesis.

However, a fundamental problem of this theory is the absence of evidence as to whether the protein precipitates are the cause or the effect of metabolic derangements in the pancreas. As you know, protein "plugs" in the pancreatic ducts of patients with pancreatitis have been recognized for decades. It has been generally thought by other workers that these plugs are a secondary phenomenon rather than an initial event.

In this regard I am interested to note that when I perform a retrograde endoscopic pancreatogram in patients with early or moderate alcoholic pancreatitis, I frequently see dilatation of the main pancreatic duct without space-occupying lesions in the lumen. How does Professor Sarles relate this to his view that dilatation of the duct system is due to obstruction by proteinaceous deposits? Is he implying that changes in the main duct represent a process distinct from that occurring in smaller branches?

This brings us back to the biggest problem of research into alcoholic pancreatitis—the absence of a satisfactory animal model. Admittedly, some of the histological features of human alcoholic pancreatitis can be reproduced in experimental animals, but there exist significant differences from the human disease (Sarles et al. 1980). Furthermore, none of the clinical features (such as recurrent episodes of acute inflammation) occur in any animal model.

Therefore, it is important to consider alternative theories to that proposed by Professor Sarles. These fall into three main groups: those involving disturbances of the sphincter of Oddi, nutritional disturbances, and direct metabolic changes (table 1).

1. Sphincteric Disturbances

(i) *Biliary-pancreatic reflux.* It has long been considered that alcohol excess could (a) cause sphincteric dysfunction favoring biliary-

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NOTE: Table appears at end of paper.

pancreatic reflux, and (b) alter the composition of the bile to render it more toxic to the pancreas. Despite some obvious attractions, this theory has tended to lose support because of the absence of a "common channel" in many patients with alcoholic pancreatitis.

(ii) *Obstruction/hypersecretion.* A traditional view has been that alcohol excess could (a) cause spasm of the sphincter of Oddi, and (b) stimulate pancreatic exocrine secretion, thus causing abnormal rises in pancreatic duct pressure. Furthermore, increased sphincteric resistance to flow results from a systemic effect of ethanol and is not dependent on local inflammatory changes (Pirola and Davis 1970). Unfortunately, at present we lack the means to test the relevance of these effects in the clinical situation.

(iii) *Duodeno-pancreatic reflux.* It has been proposed that sphincteric dysfunction due to alcohol could cause duodeno-pancreatic reflux (McCutcheon 1968). This is the only theory that provides a simple explanation for the intrapancreatic activation of enzymes (reflux into the pancreas of duodenal juice containing enterokinase). Unfortunately, we lack a satisfactory model to test this theory.

2. Nutritional Disturbances

Nutritional disturbances are common in alcoholics and pancreatitis is common in malnourished populations. Furthermore, malnourished alcoholics with pancreatic insufficiency show return of pancreatic function as their nutrition improves (Mezey et al. 1970). However, Professor Sarles and his colleagues (1965) have reported that their patients with alcoholic pancreatitis were better nourished than a carefully selected control population. Furthermore, the pancreas adapts to increased nutrient intake with enhanced enzyme output and alcohol can further augment the production of digestive enzymes (Goslin et al. 1965).

Thus it has been postulated (Pirola and Lieber 1974) that diet could influence the development of alcoholic pancreatitis in two ways. First, malnutrition might predispose to chronic degenerative changes in the pancreas. Second, good nutrition could result in an active gland with a greater potential for autodigestion and for episodes of acute inflammation.

3. Metabolic Disturbances

There is surprisingly little known about the metabolic effects of alcohol on the pancreas. Professor Sarles has described in detail the precipitation of proteins that occurs in the pancreatic juice of alcoholics. I believe that the only other major lead that we have had in recent years has been the report of Dreiling and Bordalo (1977) that deposition of fat and a reduction in zymogen granules in the acinar cells are the earliest histological changes in the pancreas of alcoholics. The presence of excess lipid in an organ rich in lipase could favor a high local concentration of free fatty acids and a risk of membrane damage. Furthermore, the earliest reported metabolic effect of ethanol on the pancreas is an increase in triglyceride synthesis (Somer et al. 1980). However, it remains to be determined whether these interesting observations have any relevance to the pathogenesis of alcoholic pancreatitis.

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Somer, J.B.; Colley, P.W.; and Pirola, R.C. Further studies of the ethanol-induced changes in pancreatic lipid metabolism. *Exp Molec Path*, 33:231-239, 1980.

Table 1. Theories Proposed for the Pathogenesis of Alcoholic Pancreatitis

1. Disturbances of the Sphincter of Oddi
   - (i) Biliary-pancreatic reflux
     - (a) Bile rendered more toxic to the pancreas by alcohol
     - (b) Bile diverted into the pancreatic duct by dysfunction of the sphincter of Oddi
   - (ii) Obstruction/hypersecretion
     - (a) Systemic effect of ethanol increases resistance to flow by the sphincter of Oddi
     - (b) Alcohol ingestion stimulates secretion
     - (c) Repeated elevations of pancreatic duct predispose to chronic damage and acute inflammation
   - (iii) Duodeno-pancreatic reflux
     - (a) Gross alcohol abuse allows reflux of duodenal contents into the pancreatic duct system
     - (b) Enterokinase activates intrapancreatic enzymes

2. Nutritional Disturbances
   - (i) Malnutrition
     Produces chronic degenerative changes in the pancreas
   - (ii) High nutritional intake
     Alcohol augments adaptive increase in enzyme production and renders the pancreas more susceptible to autodigestion

3. Metabolic Changes
   - (i) Obstruction by protein precipitates
     Alcohol causes increased secretion and precipitation of proteins in pancreatic juice, commencing in small peripheral ducts and resulting in duct obstruction and periductal inflammation
   - (ii) Disturbed lipid metabolism
     Alcohol causes changes in pancreatic lipid metabolism and increased fat deposition

Metabolism and Metabolic Effects of Ethanol*

Charles S. Lieber

Abstract

Until recently it was generally believed that diseases of the alcoholic were due exclusively to malnutrition and not to the direct toxic effects of alcohol itself. The question has been raised whether in addition to nutritional factors alcohol itself may have direct toxic effects. This paper reviews studies that showed that indeed ethanol affected the liver independently of malnutrition: both fatty liver and cirrhosis were shown to result from ethanol abuse, even in the presence of an adequate diet. Furthermore, it was found that many of the effects of alcohol could be attributed to metabolic changes associated with the oxidation of ethanol in the body.

Up to 90 percent of the ethanol absorbed is eliminated through the liver. According to Dr. Lieber, this explains, in part, the striking metabolic imbalances in the liver that are aggravated by the lack of a feedback mechanism to adjust the rate of ethanol oxidation to the metabolic state of the hepatocytes, and the inability of ethanol, unlike other major sources of calories, to be stored or metabolized to a significant degree in peripheral tissues.

Dr. Lieber describes the chain reaction of imbalances in the liver and how they impinge on the normal metabolic processes. He describes the effects of acetaldehyde, the first major "specific" oxidation product of ethanol; the role of other components of the beverage and their possible impact on acetaldehyde metabolism; the aggravation of hepatotoxicity; and the inhibition of hepatic regeneration.

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*Full article published in: *Seminars in Liver Disease*, 1(3):189-202, 1981.

SESSION II: PATHOLOGY AND IMMUNOPATHOLOGY OF ALCOHOLIC LIVER DISEASE

Alcoholic Hyalin and Alcoholic Hepatitis

Carroll M. Leevy, Natarajan Kanagasundaram, and Thomas Chen

The finding that autologous liver added to lymphocytes from patients with alcoholic hepatitis produced transformation and migration inhibitory factor (MIF) equivalent to that encountered in schistosomal liver disease, chronic active hepatitis, or biliary cirrhosis (Sorrell and Levy 1972) has clearly demonstrated the importance of immune reactivity in pathogenesis of alcoholic liver disease in man. It was postulated that alcoholic hyalin is responsible for this response, because isolates of this glycoprotein produce identical effects (Zetterman et al. 1976), whereas nuclei, microsomes, mitochondria, or membranes from reactive liver are without effect. This hypothesis is consistent with Mallory's original description (Mallory 1911) in which necrosis, inflammation, and fibrosis encountered of cirrhosis in alcoholics is attributed to alcoholic hyalin. Three lines of research have been pursued based on this postulate: (a) purification of alcoholic hyalin and delineation of the chemical characteristics of its antigenic moiety, (b) investigation of the effect of isolates of alcoholic hyalin on cell-mediated and humoral immunity, and (c) studies of factors responsible for the accumulation of alcoholic hyalin.

Purification of Alcoholic Hyalin

A variety of techniques have been used to purify alcoholic hyalin, a large amorphous complex which in initial isolates contained membranes and organelles (Okamura et al. 1975; Tinberg et al. 1978). Criteria for purity of alcoholic hyalin that is immunologically reactive now consist of the presence of Type I and/or Type II hyalin on electron microscopy without debris, MIF production when added to lymphocytes from patients with alcoholic hepatitis, and production of a specific antiserum that exhibits no reactions to membranes or nuclei. Pure, immunologically reactive alcoholic hyalin has been obtained using chemical studies that indicate it has one peak on high-pressure liquid chromatography (figure 1) and one band in the pH range between 6.1 and 6.4 with isoelectric focusing. Moreover, both

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NOTE: Figures and table appear at end of paper.

polyclonal and monoclonal antibodies have been demonstrated (Morton et al. 1981). Investigations are underway to determine its reactivity with antisera of intermediary fibrillary constituents: prekeratin (Franke et al. 1979), vimentin (Morton et al. 1980), and desmin (Hildiz et al. 1980). It has been suggested that prekeratin is the active chemical ingredient of hyaline (Denk et al. 1975); however, immunochemical studies refute this concept (Morton et al. 1980), and adding prekeratin to sensitized lymphocytes from patients with alcoholic hepatitis has no effect (Kanagasundaram and Leevy, unpublished data). The molecular weight of purified isolates of alcoholic hyalin, vimentin, and desmin are in the range of 52,000 to 57,000 daltons, which is consistent with the possible relation of hyaline to these substances (Borenfreund et al. 1980).

The relative insolubility of alcoholic hyaline in water and the need to employ SDS and urea make use of chemical isolates for immunologic studies difficult. A combination of chemical isolation procedures and the absorption of the active constituent permits production of large quantities of the pure antigenic moiety needed to determine its chemical composition and develop a radioimmunoassay to detect alcoholic hyaline in body fluids. Alcoholic hyaline antigen (AHAg) and its antibody (AHAb) were detected in sera of patients with alcoholic hepatitis using complement fixation and immune adherence hemagglutination tests (Kanagasundaram et al. 1977). These tests are negative in patients with alcoholic fatty liver, viral hepatitis, and other hepatic disorders. A radioimmunoassay for AHAb has been developed using a solid phase system that provides consistent and reproducible results (Kanagasundaram and Leevy 1979). In contrast, efforts to develop a reliable and reproducible radioimmunoassay for AHAg have not been successful to date. Studies are underway to achieve this objective utilizing a monoclonal antibody to alcoholic hyalin.

Immunologic Reactivity of Hyalin

Purified isolates of alcoholic hyalin with some nuclear and membrane debris have been used for immunologic studies. These investigations show that lymphocytes sensitized to alcoholic hyalin elaborate cytotoxic (Leevy et al. in press), fibrogenic (Chen et al. 1973), and other lymphokines in addition to MIF (Zetterman et al. 1976). Lymphocytes from patients with alcoholic hepatitis are cytotoxic when added to autologous liver, Chang liver cells (Kakumu and Leevy 1977), or rabbit liver cells (Cochrane et al. 1977). Addition of acetaldehyde augments the cytotoxicity of lymphocytes, whereas alcoholic hyalin or serum from patients with alcoholic hepatitis and circulating immune complexes block this effect (Kakumu and Leevy 1977). Cytotoxicity is exhibited by isolated T, B, and null lymphocytes (Cochrane et al. 1977); it is attributed to killer cells in the absence of adequate suppressor cells, reflected by the suboptimal response of peripheral and intrahepatic lymphocytes to concanavalin A (figure 2).

AHAg-Ab reactions appear to contribute to polymorphonuclear leukocyte chemotaxis and accumulation of neutrophils in the livers of patients with alcoholic hepatitis (Kanagasundaram et al. 1980). PML chemotaxis has been investigated using a modified Galen's double membrane technique. Chemotactic stimuli are generated in the lower compartment of Boyden chambers with complement or AH (10 mg) with 1 ml of 1/40 dilution of heat inactive RAS or 1 ml of 1/10 dilution of human serum. Homogenates of liver biopsies from patients with alcoholic hepatitis also induce chemotaxis. Preliminary studies indicate sensitized lymphocytes from patients with alcoholic hepatitis may also elaborate a chemotactic lymphokine that could be responsible for the accumulation of both neutrophils and lymphocytes during various phases of alcoholic hepatitis.

In patients with alcoholic hepatitis, leukocyte inhibitory factor (LIF) levels remain elevated until histologic improvement is noted (Kanagasundaram and Leevy in press). Highest levels occur in acutely ill subjects who exhibit anergy on skin tests and decrease in in vitro hepatic DNA synthesis. Sera from patients with alcoholic hepatitis reduce the uptake of tritiated thymidine by lymphocytes stimulated by PHA and liver cells (Hsu and Leevy 1971). Lymphocytes from patients with alcoholic hepatitis cause a significant diminution of H^3 thymidine uptake by fetal hepatocytes as compared to lymphocytes from normal subjects, suggesting AH-stimulated lymphocytes may reduce hepatic regeneration (table 1) and thereby contribute to progressive reduction in hepatic mass.

Supernatants of lymphocytes sensitized to alcoholic hyalin stimulate collagen synthesis and may be a key factor leading to progressive fibrosis and cirrhosis in alcoholics. There is a significant difference between collagen synthesis induced by supernatants of lymphocytes from patients with normal liver compared with supernatants of lymphocytes from patients with alcoholic hepatitis (Chen et al. 1976). Lymphocyte lysates from both normal subjects and patients with alcoholic hepatitis suppressed collagen synthesis. With recovery from acute phases of alcoholic hepatitis, elaboration of fibrogenic factor in the supernatant gradually subsides while the suppressor effect of lymphocyte lysates on collagen synthesis remains unchanged (figure 3). Investigations in progress are designed to characterize the chemical composition of fibrogenic and other lymphokines (Chen and Leevy, unpublished).

Mechanism of Hyalin Accumulation

Evaluation of factors responsible for the accumulation of alcoholic hyalin has consisted of immunochemical, morphologic, and epidemiologic studies. Noteworthy is the fact that although a moiety in alcoholic hyalin evokes immunologic reactivity, use of immunosuppressants in treating alcoholic hepatitis has not facilitated its dispersal. It would appear that alcoholic hyalin is produced by a variety of factors, and its accumulation represents a disparity between production and removal. Although immunochemical studies suggest that hyalin is a normal part of the cytoskeleton that is increased under adverse conditions, further studies are needed to clarify the events responsible for its accumulation and to determine whether the antigenic moiety is present under normal circumstances. This research is now feasible with pure hyalin, which is not contaminated with cell membranes and other debris. Because hyalin that is morphologically identical to that seen in alcoholic hepatitis has been found in a large number of hepatic disorders in patients who have not ingested alcohol, the role of ethanol in the production of hyalin has been questioned.

The finding of this glycoprotein in diabetes mellitus (Falchuk et al. 1980), following prolonged hyperalimentation (Craig et al. 1979), and after bypass surgery for obesity (Peters et al. 1975), has suggested that malutilization or unavailability of as yet unidentified nutrients may be the key mechanism for accumulation of hyalin in alcoholic liver disease and other states. Immunologic relationship of hyalin in these various conditions has not been established; it is hoped that studies of Griseofulvin and other animal models for production of hyalin will allow delineation of the role of nutrient imbalance in pathogenesis.

Summary and Conclusions

Alcoholic hyalin produces cell-mediated and humoral immune reactions that contribute to cytotoxicity, chemotaxis, migration inhibition, and fibrogenesis responsible for conversion of alcoholic hepatitis to cirrhosis. Understanding these reactions is important in developing effective preventive, diagnostic, and therapeutic measures for alcoholic liver disease.

Immunologically reactive alcoholic hyalin has been isolated and purified. Investigations are now underway to characterize it chemically; develop a sensitive, reproducible radioimmunoassay to detect it and its antibody in body fluids; and determine its relationship to intermediary fibrillary constituents. A fascinating area of investigation is the possibility that malutilization or deficiency of unidentified nutrients may be of key importance in the accumulation of alcoholic hyalin.

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Figure 1. Purified Isolate of Alcoholic Hyalin That Is Active as an Antigen

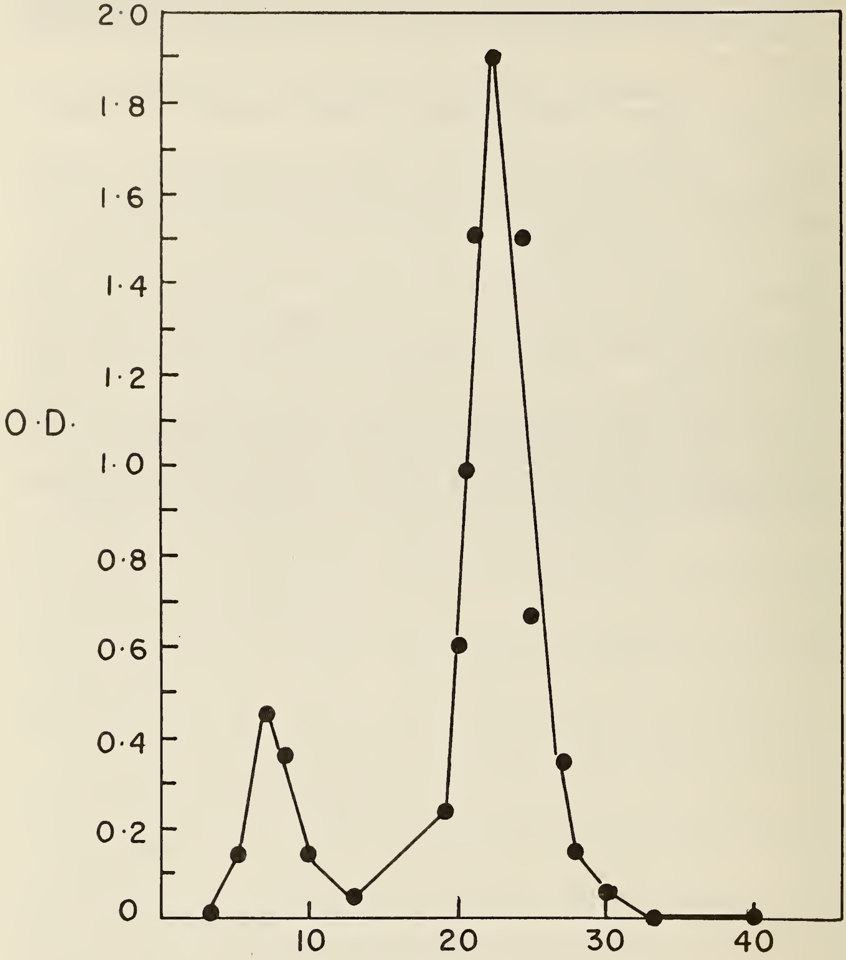

Figure 2. **Influence of PHA and Concanavalin A on Peripheral and Intrahepatic Lymphocytes from Patients with Alcoholic Hepatitis**

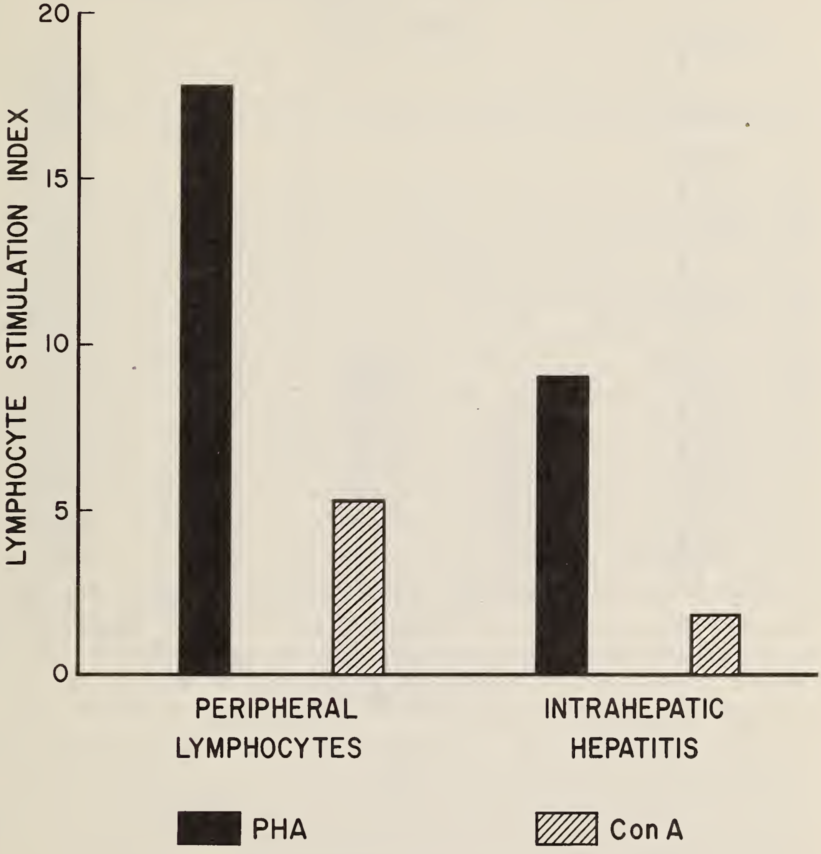

Figure 3. Influence of Supernatants of Lymphocytes on Incorporation of Tritiated Proline into Collagen by Fibroblasts

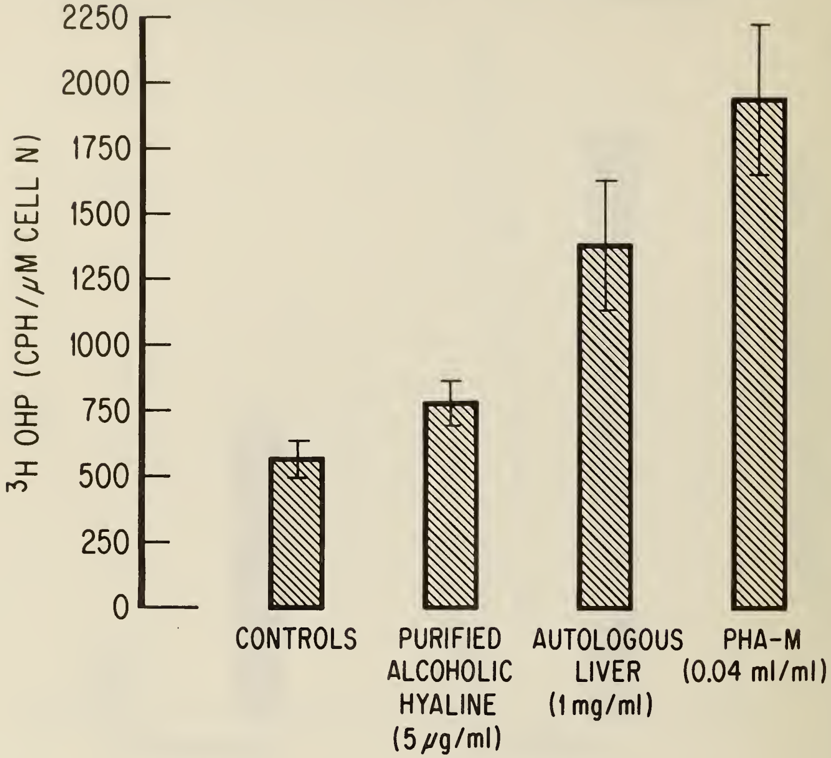

Table 1. Hepatotrophic Effect of Lymphocytes

Lymphocytes*	Fetal Rat Hepatocytes (Tritiated Thymidine Uptake into DNA, DPN/Cells × 10^5)
None	105 ± 12
Normal subjects (4)	1250 ± 145
Alcoholic hepatitis (5)	340 ± 47

*2 × 10^6 lymphocytes added to 5 days' growth of cultures of fetal rat hepatocytes × 10^5.

Effect of Chronic Ethanol Treatment on Hepatic Mitochondrial Functions in the Baboon

Masao Arai, Ellen R. Gordon, and Charles S. Lieber

The spectrum of alcoholic liver injury, from fatty liver to alcoholic cirrhosis, has been observed in baboons fed ethanol chronically (50 percent of total calories) with nutritionally adequate diet (Lieber and DeCarli 1974). This animal model, in which the disease progresses beyond the fatty liver stage, permits us to investigate the morphological and biochemical alterations as the advanced stages of injury develop. A previous study revealed that ultrastructural changes of mitochondria, e.g., enlargement, irregular form, and disoriented cristae, were seen at the fatty liver stage of chronic ethanol-fed baboons, and that these alterations persisted with the progression of the disease (Lieber et al. 1975). These morphological changes were very similar to those in chronic alcoholics. The mitochondrial functions play a central role in regulating the intermediate metabolism of the liver. Therefore, this study was intended to reveal the effect of chronic ethanol consumption on the hepatic mitochondrial functions of baboons exhibiting a fatty liver or fibrosis.

The baboons were pair-fed as described in a previous report (Lieber and DeCarli 1974). Liver was removed by surgical biopsy after an overnight fasting. Liver mitochondria were prepared according to the method of Beattie (1968), except for washing the mitochondrial pellet with medium containing 0.5 percent bovine serum albumin (fatty-acid free). Oxygen consumption was measured polarographically at 28°C utilizing a Clark oxygen electrode. The mitochondrial suspension (3-5 mg mitochondrial protein) was added to 3 ml of respiration medium containing 0.225 M sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 mM KCl, and 20 mM triethanolamine buffer (pH 7.4) (Estabrook 1967). The final concentration of substrates was 3.3 mM glutamate, 150 μM acetaldehyde, 3.3 mM succinate, and 5 mM ascorbate plus 0.2 mM N,N,N,N′=tetramethyl-p-phenylenediamine. State 3 respiration was induced by addition of 150 μM ADP. Both ADP/O ratio and respiratory control ratio (RCR) were calculated according to Estabrook (1967). Enzyme activity in the mitochondrial

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NOTE: Tables appear at end of paper.

respiratory chain was measured as follows: NADH dehydrogenase activity according to Minakami et al. (1962), succinate dehydrogenase activity according to King (1967), cytochrome c reductase activity according to Sottocasa et al. (1967), and cytochrome oxidase activity according to Wharton and Tzagoloff (1967). The concentration of cytochromes in mitochondria was determined using the method of Williams (1964). The incorporation of ^{14}C-leucine into mitochondrial membranes was measured by the method of Beattie et al. (1970).

It has been well established that electrons are supplied to the respiratory chain at the three different levels: first, at the level of NADH dehydrogenase (NAD$^+$-dependent substrates); second, at the level of flavin-linked substrates (succinate); and last, at the level of cytochrome c (ascorbate). The phosphorylation of ADP also takes place at three sites in the mitochondrial respiratory chain. The capacity to oxidize substrates and oxidative phosphorylation of ADP in mitochondria was studied using substrates entering each of the three different levels. At the fatty liver stage of chronic ethanol-fed baboons, state 4 respiration (resting respiration) was decreased with all substrates tested. Oxygen consumption was increased by the stimulation of ADP in both experimental groups, but state 3 respiration (ADP stimulated respiration) was significantly lower in mitochondria from ethanol-fed baboons (table 1). The ADP/O ratio and the respiratory control ratio were also depressed for all substrates in ethanol-fed baboons (table 2). These various alterations in functions persisted with the progression of the injury (tables 1 and 2). In association with these functional changes, the enzyme activities in the mitochondrial respiratory chain and the concentration of cytochromes were decreased in the baboon exhibiting either a fatty liver or fibrosis. The most marked changes were observed in cytochrome oxidase activity and cytochrome aa_3 contents. These changes could be related to the impairment of mitochondrial protein synthesis (table 3).

The present study suggests that chronic ethanol consumption interferes with mitochondrial membrane biogenesis in the liver and leads to the impairment of electron transfer in the mitochondrial respiratory chain. The alterations of the mitochondrial enzyme system, in turn, lead to a decrease in the phosphorylation of ADP. When these changes in mitochondrial functions are compared to those found in previous studies using a rodent model (Cederbaum et al. 1974; Bernstein and Penniall 1978; Gordon 1973), the damage appears to be more severe in the primate model. This finding is consistent with the observation that ultrastructural changes are more extensive in the primate than in the rodent (Lieber et al. 1972). The difference may be due to species difference and also to the longer term treatment with a diet containing a high concentration of ethanol

(50 percent of total calories).

In conclusion, even at a fatty liver stage of alcoholic liver injury, mitochondrial functions are markedly altered, and these changes persist with the progression of the liver disease to the fibrotic stage.

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Table 1. Oxygen Uptake of Mitochondria Isolated From Ethanol-Fed Baboons

		State 3			State 4		
			Ethanol			Ethanol	
Site	Substrate	Control	Fatty Liver	Fatty Liver and Fibrosis		Fatty Liver	Fatty Liver and Fibrosis
I	Glutamate	30.19±1.40 (9)	12.25±1.31*** (5)	15.00±1.78*** (4)	11.07±0.48 (9)	7.91±0.54*** (5)	8.58±1.03* (4)
	Acetaldehyde	27.33±1.48 (9)	15.52±0.98**** (5)	16.32±2.44*** (4)	10.36±0.69 (9)	7.91±0.17** (5)	8.90±1.03 (4)
II	Succinate	77.14±4.03 (9)	42.33±3.86**** (5)	45.46±5.22**** (4)	22.46±1.44 (9)	17.07±0.51** (5)	20.04±2.51 (4)
III	Abscorbate	96.56±6.51 (7)	47.19±5.34**** (5)	67.36±13.55 (2)	56.56±4.03 (7)	34.17±3.16*** (5)	49.76±8.54 (2)

Note: Oxygen consumption was measured polarographically. Results are expressed in nmoles O_2/min/mg protein (mean±SEM). Numbers of animals are indicated in parentheses. Significance was determined by group t-tests (compared to controls). State 3 respiration was initiated by addition of 150μM ADP.

*$p < 0.05$
**$p < 0.025$
***$p < 0.005$
****$p < 0.001$

Table 2. Effect of Chronic Ethanol Consumption on ADP/O Ratio and the Respiratory Control Ratio (RCR) Associated with the Oxidation of Glutamate, Acetaldehyde, Succinate, and Ascorbate

		ADP/O			RCR		
			Ethanol			Ethanol	
Site	Substrate	Control	Fatty Liver	Fatty Liver and Fibrosis	Control	Fatty Liver	Fatty Liver and Fibrosis
I	Glutamate	2.47±0.07 (9)	1.78±0.08** (5)	1.70±0.13** (4)	2.75±0.14 (9)	1.55±0.13** (5)	1.80±0.25* (4)
	Acetaldehyde	2.54±0.07 (9)	2.05±0.07** (5)	1.83±0.19** (4)	2.67±0.07 (9)	1.98±0.16** (5)	1.85±0.24* (4)
II	Succinate	1.77±0.03 (9)	1.53±0.05** (5)	1.41±0.04** (4)	3.48±0.15 (9)	2.49±0.23* (5)	2.32±0.25* (4)
III	Abscorbate	0.83±0.02 (7)	0.66±0.04* (5)	0.59±0.07* (2)	1.71±0.03 (7)	1.37±0.04** (5)	1.35±0.04* (2)

Note: ADP/O ratio was determined according to Estabrook (1967). The respiratory control ratio (state 3/state 4) was determined from the state 3 and state 4 respiratory rate given in table 1. Results are expressed as mean±SEM. Number of animals is indicated by parentheses. Significance was determined by group t-tests (compared to controls).

*$p<0.005$

**$p<0.001$

Table 3. Effect of Chronic Ethanol Consumption on Enzyme Activities, Cytochrome Content, and Incorporation of ^{14}C-Leucine in Baboon Hepatic Mitochondria

Experiment	Control	Ethanol	Percentage Change	p
NADH dehydrogenase (7)	1.96±0.07	1.50±0.04	−23.5	<0.001
Succinate dehydrogenase (7)	0.166±0.007	0.120±0.009	−27.8	<0.005
Cytochrome c reductase (7)				
Glutamate-	0.158±0.010	0.092±0.013	−42.0	<0.005
NADH-	0.287±0.014	0.211±0.020	−26.6	<0.01
Succinate-	0.180±0.008	0.124±0.007	−31.0	<0.001
Cytochrome oxidase (7)	1.57±0.09	0.64±0.09	−58.8	<0.001
Cytochrome content (7)				
$c + c_1$	0.263±0.015	0.162±0.010	−38.4	<0.001
b	0.220±0.007	0.137±0.007	−36.8	<0.001
aa_3	0.144±0.007	0.049±0.008	−56.9	<0.001
^{14}C-leucine incorporation (5)	4096±373	3161±192	−22.8	<0.01

Note: Enzyme activities, cytochrome content, and incorporation of ^{14}C-leucine were measured as described in text. Results are expressed as mean±SEM. NADH dehydrogenase activity is expressed as μmoles ferricyanide reduced/min./mg-protein, succinate dehydrogenase as μmoles succinate oxidized/min./mg-protein, cytochrome c reductase as μmoles ferricytochrome c reduced/min./mg-protein, and cytochrome oxidase as μmoles ferrocytochrome c oxidized/min./mg-protein. Cytochrome content is expressed as nmoles/mg-protein. ^{14}C-leucine incorporation is expressed as dpm/20 min/mg-protein. Number of animals is indicated in parentheses. Significance was determined by paired t-tests.

Pathology of Alcoholic Liver Diseases*

Hans Popper, Swan N. Thung, and Michael A. Gerber

Abstract

This paper reviews the pathology of liver disease, pointing out the few areas in the pathology where arguments prevail; describing in detail morphologic features; reviewing cellular and biochemical aspects of fibrosis as a crucial factor in prognosis, progression, and treatment; and discussing the association of alcoholic liver disease with other hepatic disorders. The authors observe that it is accepted that human alcoholic liver disease has three major stages: steatosis, hepatitis, and cirrhosis. Several questions that have not been answered involve the pathogenesis of alcoholic hepatitis, not yet reproduced in animals, and the mechanism of fibrosis in the transition to cirrhosis. The pathogenesis of fibrosis is of particular importance, the authors conclude, holding out hope for clinical parameters monitoring active fibroplasia and for antifibroblastic therapy.

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Immunologic Factors in Alcoholic Liver Diseases*

Fiorenzo Paronetto

Abstract

This paper critically reviews reports of various types of immune responses in alcoholic liver diseases and presents an hypothesis on the pathogenesis of the defects of immune response. The author discusses morphologic alterations in alcoholic liver diseases; immunologic alterations in alcoholics without liver involvement; humoral alterations in alcoholic liver diseases; immunopathologic investigations of the liver and other organs of patients with alcoholic liver diseases; cell-mediated immunity in alcoholic liver diseases; alcoholic hepatitis and chronic active hepatitis; the mononuclear-phagocytic system in alcoholic liver diseases; and genetic factors in alcoholic liver diseases. He discusses an experimental model of chronic alcoholic liver disease in the baboon and presents the hypothesis that alcohol produces liver cell alteration and, in some susceptible patients, sensitization of lymphocytes against a variety of liver antigens, one of which may be alcoholic hyalin or some other hepatic cell membrane antigen. T lymphocytes localize in the liver and this is reflected by a decrease of T cells in peripheral blood. Sensitized lymphocytes produce either direct cytotoxicity of a variety of lymphokines which may amplify liver cell damage, induce chemotaxis or neutrophils, and stimulate fibrogenesis. A concomitant alteration takes place in the organism. The author concludes that further research may provide a more precise understanding of the causal role of immune reactions in alcoholic liver disease, and lead to appropriate immunotherapy that could alter the development of these diseases.

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SESSION III: ACTIVATION OF HEPATOTOXIC AGENTS AND CARCINOGENS

Activation of Hepatic Toxic Agents and Carcinogens: An Introduction

Paul Sherlock

In North America and most of Europe, it appears that alcohol consumption and smoking are the most important determinants of oral cavity and esophageal cancer. Alcohol may function as a direct irritant or may be associated with a nutritional deficiency resulting in chronic irritation. Tobacco smoking makes an independent contribution to the development of esophageal and oral cavity cancers, and the use of alcohol by cigarette smokers significantly increases the risk for these cancers. The mucosa conditioned by nutritional deficiency may be particularly vulnerable to the action of multiple carcinogens.

There are other possible explanations, however. Chronic ethanol consumption results in induction of microsomal enzymes, which may be associated with an increased capacity to activate procarcinogens to carcinogens. It is also possible that the synergism seen between alcohol consumption and smoking with certain types of cancers is related to the induction of microsomal enzymes and metabolic activation of smoke carcinogens.

The paper by Seitz et al. explores the effect of chronic ethanol feeding on the activation of procarcinogens to their mutagenic derivatives as well as the activation of compounds present in tobacco pyrolyzates. The paper by McCoy et al. demonstrates that chronic ethanol consumption increases the sensitivity of the nasal and tracheal mucosa to the carcinogenic effect of N-nitrosopyrollidine present in tobacco.

The paper by Teshke, however, demonstrates that although chronic ethanol administration favors the toxifying metabolic pathway for chlorpromazine and paracetamol, it enhances the detoxifying pathway for dimethylnitrosamine.

Another problem encountered in clinical medicine is halothane hepatic toxicity. In experiments attempting to delineate the mechanism, rats fed ethanol and receiving halothane at low oxygen conditions showed multifocal necrosis. This finding raises the possibility that chronic ethanol ingestion in humans may predispose to halothane hepatic damage, particularly if the conditions at the time of surgery result in hypoxia.

As these papers demonstrate, alcohol may play a crucial role in affecting the microsomal enzyme system, which is important in the activation of hepatotoxic agents and carcinogens.

Increased Activation of Procarcinogens by Microsomes of Various Tissues Induced by Chronic Ethanol Ingestion*

Helmut K. Seitz, Anthony J. Garro, and
Charles S. Lieber

Summary

Chronic ethanol consumption in rats results in an increase in microsomal cytochrome P-450 of the liver, intestine, and lungs. This increase is associated with an enhanced capacity of hepatic, intestinal, and pulmonary microsomes to activate different types of procarcinogens to their mutagenic derivatives using a Salmonella typhimurium auxotroph reversion test.

In the liver, the activation of dimethylnitrosamine to a mutagen by microsomes is significantly enhanced after alcohol feeding. This is observed in both male and female rats. In contrast, hepatic microsomal activation of benzo(a)pyrene is enhanced only in females after ethanol ingestion. The ethanol feeding also increases the capacity of intestinal microsomes to activate to mutagens tryptophan pyrolyzate, benzo(a)pyrene, and 2-aminofluorene. Subsequently, pulmonary microsomal activation of compounds present in tobacco pyrolyzate to mutagens for Salmonella typhimurium is enhanced by chronic ethanol consumption.

Thus, chronic ethanol ingestion influences carcinogen metabolism in the liver, intestine, and lungs in a manner that varies with respect to the animal's sex, the carcinogen, and tissue. An enhanced microsomal activation of dietary and environmental carcinogens in various tissues after chronic ethanol intake may contribute to the increased incidence of cancer seen among alcoholics.

NOTE: Tables appear at end of paper.

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Introduction

Alcoholism has been associated with an increased incidence of various cancers, especially cancer of the upper alimentary and respiratory tract and the liver (Lieber et al. 1979; U.S. Department of Health, Education, and Welfare 1975; Williams and Horn 1977; Wynder et al. 1977). One of the mechanisms by which heavy alcohol consumption may affect carcinogenesis is by enhancing the metabolic activation of inhaled and ingested procarcinogens.

Chronic ethanol consumption in both humans and rats is known to increase microsomal cytochrome P-450 content and microsomal enzyme activities in the liver (Lieber 1973). An inducible cytochrome P-450 dependent biotransformation system has been demonstrated in the respiratory (Abramson et al. 1977; Hutton and Hackney 1975) and in the gastrointestinal tract (Chhabra et al. 1974; Wattenberg 1975) of several species. Recently it was reported that chronic ethanol consumption increases microsomal cytochrome P-450 content in the small intestine (Joly and Hetu 1980; Seitz et al. 1978).

Because microsomal enzymes also are involved in the activation of a variety of dietary and environmental procarcinogens, tissues such as lungs and intestine—major portals of entry into the body—and the liver, with its great metabolic capacity, may be considered as important organs with respect to carcinogen activation after alcohol consumption. Lungs and intestine are exposed to a relatively high concentration and a wide variety of dietary procarcinogens, although the relative metabolic capacity of these tissues is small when compared with that of the liver (Chhabra 1974). In addition, different procarcinogens, such as polycyclic hydrocarbons (Masuda et al. 1966), nitrosamines (Lijinsky and Epstein 1970), and asbestos fibers (Cunningham and Pontefract 1971) have been detected in small amounts in alcoholic beverages.

In the present study, the effect of chronic ethanol ingestion on the activation of different types of procarcinogens by hepatic, intestinal, and pulmonary microsomes was investigated using the Ames Salmonella auxotroph reversion test.

Methods

Adult male Sprague Dawley rats were pair-fed adequate liquid diets containing 36 percent of total calories as either ethanol or isocaloric carbohydrates (Lieber and DeCarli 1970). Following 3 to 4 weeks of feeding, the rats were sacrificed after an overnight fast. Microsomes were prepared from the liver (Czygan et al. 1973), from the mucosa of an intestinal segment consisting of the first 25 cm distal to the pylorus (Stohs et al. 1976), and from the lungs (Seitz et al. in press). The microsomal suspensions were filtered through Milipore filters (SXHA 025 OS Swinnex units) to remove contaminating bacteria. The protein content of the microsomes was measured according to the method of Lowry et al. (1951). Cytochrome P-450 content was determined from the CO bound difference spectrum by the method of Omura and Sato (1964).

Benzo(a)pyrene hydroxylase activity was measured by the method of Dehnen et al. (1973) using 3-hydroxybenzo(a)pyrene as a standard. Microsomal activation of procarcinogens/promutagens to their mutagenic derivatives was assayed with either the Salmonella typhimurium His$^-$ strain TA 100 or with the His$^-$ strain TA 1530 as described by Ames et al. (1975), using, however, microsomes instead of the 9,000 g supernatants of the homogenized tissues (Guttenplan et al. 1976). To measure dimethylnitrosamine induced mutagenesis at dimethylnitrosamine concentrations in the range of 0.3-1.0 mM, the bacteria were pretreated with nitrosomethylurea as suggested by Guttenplan prior to being exposed to dimethylnitrosamine (Guttenplan 1979).

All the mutagenesis assays were performed in duplicate, and the number of His$^+$ colony formed units on duplicate plates generally were within 10 percent of each other. Within individual experiments, the standard deviation of the observed spontaneous mutation frequency in the absence of added mutagen was ±10 percent.

The carcinogens benzo(a)pyrene and 2-aminofluorene were purchased from the Aldrich Chemical Company Inc. Dimethylnitrosamine was purchased from the Eastman Kodak Co. The tryptophan and tobacco pyrolyzates were prepared by heating the material in evacuated sealed glass ampules at 500°C for 1 min as described by Matsumoto et al. (1978). The pyrolyzates were dissolved in spectrophotometric grade dimethyl sulfoxide obtained from Schwarz/Mann.

Results

Chronic ethanol consumption resulted in a significant increase in cytochrome P-450 content of the mucosa of the small intestine and of the lungs (table 1). In the intestine, this increase was associated with a parallel increase in microsomal benzo(a)pyrene hydroxylase activity, whereas no such increase occurred in the lungs (table 1).

In the liver, a sex-dependent effect of chronic ethanol ingestion on microsomal benzo(a)pyrene hydroxylase activity was found. In female rats, ethanol administration resulted in a significant enhancement of hepatic microsomal benzo(a)pyrene hydroxylase activity, whereas in males no such difference was seen (table 2). This finding was paralleled by the observation that chronic ethanol ingestion increased the ability of hepatic microsomes from female rats to activate benzo(a)pyrene to a mutagen but not from male rats (table 3).

In contrast, hepatic microsomal dimethylnitrosamine demethylase activity was stimulated after chronic ethanol consumption both in males and in females at all dimethylnitrosamine concentrations studied (table 2). In addition, a significantly enhanced capacity of hepatic microsomes to activate dimethylnitrosamine to derivatives mutagenic for Salmonella typhimurium was observed (table 3).

In the intestine, ethanol increases the capacity of mucosal microsomes to activate benzo(a)pyrene, 2-aminofluorene, and tryptophan pyrolyzate to mutagens, whereas in the lungs no such differences after alcohol administration occurred (table 4). However, pulmonary microsomes from chronically ethanol-fed rats activated tobacco pyrolyzate to mutagen(s) to a significantly higher degree than did microsomes from their pair-fed controls (table 4).

Discussion

The results show that chronic ethanol consumption significantly affects intestinal, hepatic, and some pulmonary microsomal enzyme activities and that this effect influences the capacity of these tissues to activate potentially carcinogenic compounds.

The enhanced hepatic activation of the potent carcinogen dimethylnitrosamine after ethanol seems of special interest, since dimethylnitrosamine was found to be present in alcoholic beverages (Lijinsky and Epstein 1970; Walker et al. 1976). It is particularly noteworthy that these effects of ethanol ingestion are detectable in vitro at dimethylnitrosamine concentrations in the range of 0.3 to 1 nM. These dimethylnitrosamine conditions are sufficiently low to suggest that chronic ethanol consumption may enhance the in vivo metabolic activation of dimethylnitrosamine at dimethylnitrosamine concentrations that may be pathologically relevant.

Alcohol also significantly increases the capacity of pulmonary microsomes to activate mutagens present in tobacco pyrolyzate. This observation is particularly interesting in light of the synergistic effect of ethanol consumption and cigarette smoking on carcinogenesis (U.S. Department of Health, Education, and Welfare 1975; Wynder et al. 1977). It has in fact been suggested that the respiratory tract is the primary site of metabolic activation of the various promutagens present in cigarette smoke (Kier et al. 1974). Cigarette smoke condensates are known to contain a mixture of potentially mutagenic compounds (Kier et al. 1974) and tobacco pyrolyzates are presumably similar in this regard.

It recently has been shown in hamsters that dietary ethanol also enhances the capacity of liver microsomes to activate another mutagenic compound found in tobacco smoke, nitrosopyrollidine (McCoy et al. 1979).

Chronic ethanol ingestion also increases intestinal microsomal activation of the polycyclic hydrocarbon benzo(a)pyrene, of 2-aminofluorene, and tryptophan pyrolyzate. Both benzo(a)pyrene and the pyrolysis products of tryptophan are found in charcoal broiled meats and fish and as such may be considered potential dietary carcinogens. Sugimura et al. (1977) recently identified two mutagens in tryptophan pyrolyzate, namely 3-amino-1, 4-dimethyl-5H-pyrido (4,3-6)-indole and 3-amino-1-methyl-5H-pyrido(4,3-6)-indole. Actually, these two compounds have been found to be more mutagenic in the Salmonella system than has the well-known dietary carcinogen aflatoxin B1 (Nagao et al. 1978).

Small bowel tumors are rare, but with respect to carcinogenesis the small intestine may be considered as a site of carcinogen production rather than a target site for activated carcinogens. Depending on the half-life time, carcinogens could be produced in a site distant of their action, as has been demonstrated for phenyltriazene, which is metabolically activated in the liver but causes brain tumors (Bartsch et al. 1977).

It is noteworthy that in various animal experiments ethanol has been shown to have a cocarcinogenic effect when administered in conjunction with a number of different procarcinogens, including benzo(a)pyrene (Horie et al. 1965), 7,12-dimethyl-benzanthracene (Elzay 1966), dimethylnitrosamine (Giebel 1967), and vinylchloride (Radike et al. 1977).

The fact that dietary ethanol can influence microsomal enzyme activity in a variety of tissues is of particular significance considering the widespread use of alcoholic beverages. It should be noted that the level of alcohol used in this study, 36 percent of total calories, leads to a blood ethanol concentration of that seen in social drinkers (Lieber et al. 1965).

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Table 1. Effect of Chronic Ethanol Consumption on Microsomal Cytochrome P-450 Content and Microsomal Benzo(a)pyrene Hydroxylase Activity in Intestine and Lungs

Feeding	Cytochrome P-450 (pmoles/mg micr.protein)		Benzo(a)pyrene Hydroxylase Activity (pmoles 3-OHBP/mg micr.protein/min)	
	Intestine (n=7)	Lungs (n=9)	Intestine (n=7)	Lungs (n=5)
Ethanol-fed Rats*	26.0±4.6	38.3±5.3	18.1±3.3	22.4±3.2
	$p<0.01$	$p<0.01$	$p<0.01$	NS^{+}
Pair-fed Controls*	8.9±2.1	24.9±3.0	6.6±1.0	21.5±3.2

* Values are mean±SEM. + Not statistically significant ($p>0.05$).

Table 2. Effect of Chronic Ethanol Consumption on the Microsomal Activity of Benzo(a)pyrene (BP) Hydroxylase and Dimethylnitrosamine (DMN) Demethylase in the Liver of the Rat

Feeding	BP-Hydroxylase (pmoles 3-OHBP/mg micr.prot./min)		DMN-Demethylase (nmoles formaldehyde/mg micr.prot./min)			
			(100 mM)*		(5 mM)*	
	Male (n=7)	Female (n=7)	Male (n=5)	Female (n=5)	Male (n=5)	Female (n=5)
Ethanol-fed Rats^{+}	720±198	128±7	4.0±0.2	6.0±0.1	1.6±0.1	1.8±0.1
	NS§	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$	$p<0.01$
Pair-fed Controls^{+}	875±235	90±3	2.4±0.3	4.3±0.2	1.1±0.1	1.1±0.1

* Concentration of dimethylnitrosamine in the incubation system. + Values are mean ± SEM. § Not statistically significant ($p>0.05$).

Table 3. **Effect of Chronic Ethanol Consumption on the Microsomal Activation of Benzo(a)pyrene (BP) and Dimethylnitrosamine (DMN) to Mutagens in the Liver**

Feeding	BP/Plate (μg/plate)	His$^+$ Revertants/Plate*		DMN Concentration (mM)	His$^+$ Revertants/Plate*
		Male$^+$	Female^{++}		Male^{+++}
Ethanol-fed Rats	1.6	90	85	0.3	190
	3.1	150	105	0.6	520
	6.3	195	125	1.2	1400
	12.5	255	175		
	25.0	300	165		
Pair-fed Controls	1.6	115	45	0.3	50
	3.1	180	55	0.6	220
	6.3	205	100	1.2	850
	12.5	255	120		
	25.0	245	110		

* Numbers are averages of His$^+$ colonies of Salmonella typhimurium TA 100 (for BP) and of Salmonella typhimurium TA 1530 (for DMN) on duplicate plates. The numbers of spontaneous His$^+$ revertants have been subtracted.

$^+$ Microsomal protein/plate was 0.4 mg per plate.

$^{++}$ Microsomal protein/plate was 0.5 mg per plate. Similar results were obtained with 0.4-1.4 mg micr.protein plate.

$^{+++}$ Microsomal protein/plate was 2 mg per plate. The incubation time was 15 minutes.

Mutagenesis assays were performed with microsomes from pools of three animals each.

Table 4. Effect of Chronic Ethanol Consumption on the Capacity of Intestinal and Pulmonary Microsomes to Activate Various Promutagens/Procarcinogens*

Tissue	Feeding	Benzo(a)pyrene		Tryptophan Pyrolyzate		Tobacco Pyrolyzate^{+++}	
		μg/plate	His^{+} revert.§	amount/plate (μg tryptophan)	His^{+} revert.§	amount/plate (mg tobacco)	His^{+} revert.§
Intestine^{+}	Ethanol-fed Rats	1.2	40	62.5	50	No mutagenic effect detectable	
		2.5	109	125.0	101		
		5.0	125	250.0	198		
		10.0	134				
		20.0	130				
	Pair-fed Controls	1.2	11	62.5	25		
		2.5	41	125.0	49		
		5.0	54	250.0	100		
		10.0	82				
		20.0	80				

Table 4. Effect of Chronic Ethanol Consumption on the Capacity of Intestinal and Pulmonary Microsomes to Activate Various Promutagens/Procarcinogens*—Continued

Tissue	Feeding	Benzo(a)pyrene		Tryptophan Pyrolyzate		Tobacco Pyrolyzate[+++]	
		μg/plate	His$^+$ revert.§	amount/plate (μg tryptophan)	His$^+$ revert.§	amount/plate (mg tobacco)	His$^+$ revert.§
Lungs[++]	Ethanol-fed Rats	1.6	55	83	29	0.5	110
		3.1	78	167	98	1.0	240
		6.3	101	333	160	2.0	483
		12.5	110	666	168		
		25.0	113				
	Pair-fed Controls	1.6	53	83	26	0.5	62
		3.1	75	167	100	1.0	118
		6.3	99	333	157	2.0	181
		12.5	109	666	165		
		25.0	114				

[+] Microsomal protein/plate was 0.4 mg. Similar results were obtained with 0.5, 1.0, and 1.4 mg microsomal protein per plate.

[++] Microsomal protein/plate was 0.7 mg. Similar results were obtained with 0.9 and 1.1 mg microsomal protein per plate.

[+++] Microsomal protein/plate was 1.1 mg.

§ Numbers are averages of His$^+$ colonies of Salmonella typhimurium TA 100 on duplicate plates. The number of spontaneous His$^+$ revertants have been subtracted.

Influence of Chronic Ethanol Consumption by Hamsters on the Carcinogenicity of N-Nitrosopyrrolidine and N′-Nitrosonornicotine*

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Summary

Epidemiological studies have shown that the combination of chronic alcohol and tobacco consumption substantially increases the risk for cancers of the head and neck in humans. This synergism might be due to ethanol induction of metabolic activation pathways for tobacco smoke carcinogens. We have previously shown that the metabolic activation via α-hydroxylation of N-nitrosopyrrolidine (NPYR) as well as its mutagenicity is increased by liver fractions isolated from ethanol-consuming hamsters. This treatment also increases the in vitro α-hydroxylation of the tobacco-specific carcinogen N′-nitrosonornicotine (NNN). These results indicated that chronic alcohol consumption (CEC) might increase the carcinogenicity of NPYR and NNN.

To test this hypothesis, the effect of CEC on the carcinogenicity of NPYR and NNN in male Syrian golden hamsters (SGH) was investigated. Animals were given control or ethanol-containing liquid diets and then treated intraperitoneally with two dose levels (total 1 mmol or 2 mmol) of NPYR or NNN, three times weekly for 25 weeks. Mortality after 15 months was significantly greater in the ethanol-treated NPYR groups than in the control NPYR groups; these differences were not apparent with NNN. In the NPYR groups, tumors of the liver, trachea, and nasal cavity were observed, whereas NNN affected trachea and nasal cavity. At the lower dose of NPYR, ethanol-consuming SGH developed more nasal cavity and tracheal tumors than did controls. This effect was also observed at a higher dose of NPYR. No increased tumor incidence in ethanol-consuming animals

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NOTE: Figure and table appear at end of paper.

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treated with NNN was observed at either dose level. These data indicate that CEC increases the sensitivity of both the nasal and tracheal mucosa to the carcinogenic effect of NPYR and that ethanol-associated changes in microsomal metabolism may be one mechanism by which chronic alcohol consumption can increase tumor incidence.

Epidemiology of Alcohol-Related Cancers

Extensive epidemiological evidence has been available for some time now that indicates that the combination of both chronic alcohol and tobacco consumption greatly increases the risk for cancers in the head and neck area, specifically of the oral cavity, esophagus, and larynx (McCoy and Wynder 1979). The epidemiological study of the apparent interaction of smoking and drinking in head and neck cancer is complicated by difficulty in assessing these two variables independently. Because few heavy drinkers are nonsmokers, the role of alcohol consumption is difficult to assess. Even though some increased risk for cancer due solely to alcohol consumption is suggested by the data of Rothman and Keller (1972), a marked synergy is observed when tobacco use is combined with chronic alcohol consumption. With the possible exception of esophageal cancer in France (Tuyns and Masse 1973; Tuyns et al. 1977), the nature of the beverage consumed seems to be important only in terms of its ethanol content (Williams and Horn 1977; Wynder and Stellman 1977).

Occurrence and Carcinogenicity of NPYR and NNN

The nitrosamines, N-nitrosopyrrolidine (NPYR) and N′-nitrosonornicotine (NNN), are important environmental carcinogens. NPYR has been detected in bacon and other processed meats, as well as in mainstream and sidestream tobacco smoke (Brunnemann et al. 1977; Havery et al. 1978). NNN is one of the major tobacco-specific carcinogens, occurring in tobacco and tobacco smoke in relatively high concentrations. Oral administration of NPYR gives liver tumors in rats (Greenblatt and Lijinsky 1972*b*) and lung adenomas in strain A mice (Greenblatt and Lijinsky 1972*a*). Oral administration of NNN causes esophageal tumors in rats, whereas s.c. injection gives nasal cavity tumors (Hecht et al. 1980; Hoffmann et al. 1975). Subcutaneous administration to hamsters gives nasal cavity and tracheal tumors (Hilfrich et al. 1977).

In Vitro Metabolism of NPYR and NNN

α-Hydroxylation as a likely activation mechanism for nitrosamines was first suggested by Druckrey et al. (1967). The role of α-hydroxylation in the microsomal activation of nitrosamines, such as NPYR and NNN, has been supported by structure-activity studies in which decreased carcinogenicity was observed when methyl groups or deuterium atoms were substituted in the α-positions (Lijinsky and Taylor 1976; Lijinsky et al. 1976) and by the high mutagenicity, without activation of α-acetoxy cyclic nitrosamines and related model compounds (Baldwin et al. 1976; Chen, Hecht, and Hoffman 1978). Despite the apparent importance of metabolic α-hydroxylation as an activation step for NNN and NPYR, it was not until recently that methods became available for in vitro determination of these activities (Chen, McCoy, Hecht, Hoffman, and Wynder 1978; Chen et al. 1979). The mechanism for α-hydroxylation of NPYR and NNN is outlined in figure 1.

Microsomal hydroxylation of either α-carbon of NPYR gives rise to α-hydroxyNPYR, an unstable intermediate, which undergoes ring opening and loss of N_2 and hydroxide to give the 4-oxobutylcarbonium ion, which may be the ultimate carcinogenic form of NPYR. This ion reacts with water to give 4-hydroxybutyraldehyde, which exists predominantly as the cyclic hemiacetal 2-hydroxytetrahydrofuran. 4-Hydroxybutyraldehyde can be trapped as its 2,4-dinitrophenylhydrazone derivative (Chen, McCoy, Hecht, Hoffman, and Wynder 1978; Chen et al. 1979).

Because the two α-carbons of NNN are not equivalent, microsomal α-hydroxylation results in the formation of two products, a keto alcohol from 2′-hydroxylation and a lactol from 5′-hydroxylation. The products can be trapped as the corresponding 2,4-dinitrophenylhydrazones. The resultant dinitrophenylhydrazones can then be quantitated by high-pressure liquid chromatography.

Effect of Chronic Ethanol Consumption on Liver Microsomal Metabolism

It has been well established that one of the adaptive responses to chronic ethanol consumption in both experimental animals and humans is the proliferation of liver-smooth endoplasmic reticulum and marked increases in many of the enzymatic components of the mixed function oxidase system. Increases in the specific activity of aniline hydroxylase, NADPH cytochrome c reductase, as well as the content of cytochrome P-450, first observed by Rubin et al. (1968), have now been confirmed by numerous other investigators (Lieber and DeCarli 1977). The increase in cytochrome P-450 content has been shown to be due to the appearance of a hemoprotein with distinct spectral and catalytic properties (Comai and Gaylor 1973; Joly et al. 1977; Ohnishi and Lieber 1977).

The observation that like aniline the cyclic nitrosamines, N-nitrosopyrrolidine and N′-nitrosonornicotine (Chen, McCoy, Hecht, Hoffman, and Wynder 1978; McCoy et al. 1979), exhibit type II spectra suggested the possibility that the microsomal metabolism of these two compounds might also be increased as a result of chronic ethanol consumption.

Therefore, the effect of chronic ethanol consumption by hamsters on the activity of isolated liver fractions to α-hydroxylate NPYR and NNN was studied. Microsomes from ethanol-consuming hamsters exhibited increased ratio of α-hydroxylation of NPYR. In addition, increases in the specific activities of aniline hydroxylase, NADPH cytochrome c reductase, and the specific content of cytochrome P-450 were also observed. In contrast, no differences in specific activities of benzo(a)pyrene hydroxylase or glucose 6-phosphatase were observed. Liver postmitochondrial supernatants from ethanol-consuming animals were able to produce five times the number of mutants from NPYR than from control preparations (McCoy et al. 1979). Chronic ethanol consumption by hamsters also increased the specific activity of 5′ but not 2′-hydroxylation of NNN (McCoy et al. 1980). Increased rat liver microsomal dimethylnitrosamine demethylase activity following both acute and chronic ethanol consumption has also been documented (Garro et al. 1981; Maling et al. 1975; Schwartz et al. 1980).

Effect of Chronic Ethanol Consumption on the Carcinogenicity of NPYR and NNN

Because the in vitro data suggested that ethanol consumption by hamsters could increase the metabolic activation of NPYR and NNN, the effect of chronic ethanol consumption by hamsters on the carcinogenicity of these two nitrosamines was examined (McCoy, Hecht, Katayama, and Wynder in press).

Animals (21 per group) were placed on control or ethanol-consuming diets at 9 weeks of age. After 1 month, carcinogen treatment was begun and dietary administration of ethanol was continued. Carcinogens were administered three times weekly for 25 weeks, resulting in a total dose of 2 mmol (high dose) or 1 mmol (low dose).

Table 1 presents the carcinogenicity data for NPYR and NNN. The major sites of tumors in NPYR- and NNN-treated animals were the nasal cavity and trachea. Ethanol-consuming animals receiving 1 mmol NPYR had a higher incidence of both nasal cavity and tracheal tumors compared with carcinogen-treated controls. Animals receiving 2 mmol NPYR had more tumors at these two sites, but the enhancing effect of ethanol was less pronounced. Seventy percent of the nasal cavity tumors occurred in animals that were sacrificed prior to termination. The first nasal cavity tumor was observed in an animal sacrificed in experimental month 10, with the majority being observed in animals sacrificed in experimental months 14 and 15. Approximately half of the tracheal tumors occurred prior to termination. The first was seen in experimental month 10.

NNN-treated animals exhibited more tracheal than nasal cavity tumors; however, no increased incidence was observed in the ethanol-consuming groups. Approximately 60 percent of the total nasal cavity tumors appeared in NNN-treated animals sacrificed prior to termination, whereas only 15 percent of the tracheal tumors were found prior to termination. The first nasal cavity and tracheal tumors were observed in animals sacrificed in experimental months 14 and 15, respectively.

Neoplastic liver nodules were observed only in carcinogen-treated animals and occurred frequently in NPYR-treated animals. Adrenal tumors were found in all groups, and their appearance was independent of both carcinogen and ethanol.

Discussion

The results presented here indicate that chronic alcohol consumption can increase the incidence of nasal cavity and tracheal tumors in hamsters exposed to NPYR. The fact that the enhancing effect of ethanol is greater at the lower level of NPYR suggests that ethanol consumption, in some manner, increases the susceptibility of the nasal and tracheal mucosa to NPYR. Since our studies have shown that ethanol consumption increases liver microsomal metabolism and mutagenicity of NPYR (McCoy et al. 1979), it is possible that similar changes are occurring in the target tissues. The possible involvement of enhanced target tissue metabolism in ethanol-treated hamsters is currently under investigation. Ethanol by itself did not increase tumor incidence or change the organotropism of either NPYR or NNN, indicating that in this system, ethanol is not a carcinogen and that the increased tumor incidence in the target tissues is not caused by decreased incidence at some other site. It is also important to note that no tracheal or nasal cavity tumors were observed in the noncarcinogen-treated animals.

In view of the structural similarity of NPYR and NNN, the lack of an effect of ethanol consumption on the carcinogenicity of NNN was somewhat surprising. Our studies have shown that, like NPYR, the liver microsomal metabolism of NNN is increased by ethanol consumption. Unlike NPYR, however, the two sites of α-hydroxylation of NNN are not equivalent and, although the major site of in vitro hydroxylation (5′) is the site that is increased by ethanol, it is possible that 2′-hydroxylation is involved in carcinogenesis and that 5′-hydroxylation is a detoxification pathway. There are species differences in the response of 5′- and 2′-hydroxylation of NNN to induction. In the hamster liver it is 5′-hydroxylation which is inducible, whereas in the rat it is 2′-hydroxylation that can be induced (McCoy, Chen, and Hecht in press).

If 2′-hydroxylation is involved in the metabolic activation of NNN, the rat may be a more suitable model in which to examine the influences of chronic ethanol consumption of the carcinogenicity of NNN. We are currently in the process of examining the effect of chronic ethanol consumption on male Fischer 344 rats to determine if, like the other inducers of drug metabolism, ethanol will enhance the rate of 2′-hydroxylation of NNN.

The results of this study indicate that the epidemiological association of heavy smoking and consumption of alcohol with increased risk for cancers of the head and neck can be mimicked in an animal model. Further studies are needed to establish if enhanced metabolic activation is the reason for increased carcinogenicity of NPYR in hamsters; nevertheless, insight into this aspect as well as other possible mechanisms by which ethanol consumption increases the risk for cancer can be successfully studied in this model system.

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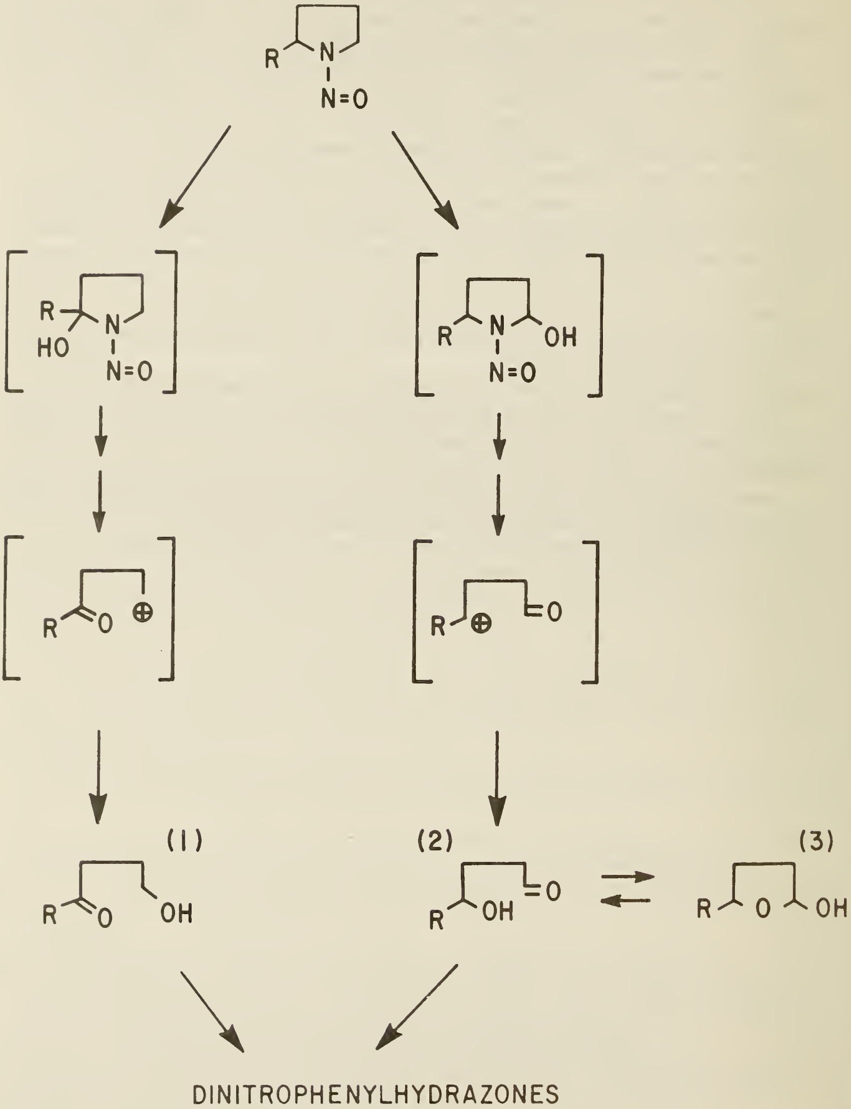
Figure 1. Mechanism of α-Hydroxylation of NPYR and NNN



Hydroxylation of NPYR (R=H) at either α-carbon gives rise to 4′-hydroxyburyraldehyde (2), which is in equilibrium with the cyclic hemiacetal 2′-hydroxytetrahydreturan (3). Hydroxylation NNN (R=3 pyridyl) gives rise to two products: the keto alcohol (1) from 2′-hydroxylation and the lactol (3) from 5′-hydroxylation. Products (1) and (2) can be trapped as the 2,4′-dinotrophenylhydrazone derivatives.

Table 1. The Influence of Chronic Ethanol Consumption on the Carcinogenicity of N-Nitrosopyrrolidine (NPYR) and N′-Nitrosonornicotine (NNN) in Male Syrian Golden Hamsters

	Experimental Conditions				Animals with Upper Respiratory Tract Tumors			
	Carcinogen							
Group	Type	Dose	Diet	Effective Animals[a]	Nasal Cavity	Trachea	Both Sites	Either Site
1	NPYR	1 mmol	Control	20	1 (0)[c]	4	1	4
2	NPYR	1 mmol	Ethanol	18	8[b](6)	9[b]	4	13[b]
3	NPYR	2 mmol	Control	21	14 (8)	8	7	15
4	NPYR	2 mmol	Ethanol	17	16[b](9)	11	10	17[b]
5	NNN	1 mmol	Control	21	1 (1)	4	0	5
6	NNN	1 mmol	Ethanol	17	1 (0)	5	0	6
7	NNN	2 mmol	Control	21	5 (2)	9	1	13
8	NNN	2 mmol	Ethanol	21	4 (1)	7	1	10
9	None	0	Control	21	0	0	0	0
10	None	0	Ethanol	19	0	0	0	0

[a] Effective animals are defined as those animals that survived the carcinogen administration period.
[b] Significantly different ($p \leq 0.05$) from control.
[c] Number of tumors with invasive characteristics.

Halothane-Induced Hepatic Necrosis in Ethanol-Pretreated Rats*

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Masaharu Tsuchiya**

Introduction

Despite much controversy over the existence of halothane-induced hepatitis, a consensus has been reached that it is a distinct although rare syndrome (Dienstag 1980). However, the mechanism by which halothane causes hepatic injury in humans is still unclear. Recently, administration of enzyme-inducing agents, such as phenobarbital, to rats in combination with hypoxic state has been reported to enhance the hepatotoxicity of halothane, resulting in centrolobular necrosis (McLain et al. 1979; Ross et al. 1979).

Since ethanol is known to produce hepatic microsomal induction in both humans and rats (Ishii et al. 1973; Lieber 1973; Rubin and Lieber 1968), we wondered if chronic ethanol administration modifies the effect of halothane on the liver.

In this paper, we report that halothane causes centrilobular hepatic necrosis in rats fed ethanol chronically, especially under hypoxic conditions.

Method

Male Wistar rats weighing approximately 200 g were maintained on a 12-hour dark and light cycle. Then the animals were housed in individual wire-bottom cages. They were fed a nutritionally adequate liquid diet (DeCarli and Lieber 1967) for 6 weeks. For the control rats, carbohydrates, protein, and fat provided 47 percent, 18 percent, and 35 percent, respectively, of the total calories. Pair-fed rats consumed the same diet except that carbohydrates were isocalorically replaced by ethanol, accounting for 36 percent of the total calories.

NOTE: Figures and table appear at end of paper.

* This research was supported in part by a grant from the Japanese Ministry of Education.

After 6 weeks of ethanol feeding, the rats were placed into Plexiglas chambers in groups of four per cage. These animals were anesthetized for 2 hours with 1 percent halothane at two different concentrations of oxygen, low (10 percent) and ambient (35 percent). The halothane and oxygen were delivered to the chamber by a Flotec III at a flow rate of 4 liters per minute. Halothane concentration was determined by Flotec III, and the chamber oxygen concentration was monitored by Teledyne oxygen analyzer type 331. Another group of pair-fed animals was treated with hypoxic condition of 10 percent oxygen concentration for 2 hours without exposure to halothane. Twenty-four hours after halothane anesthesia, the animals were sacrificed and the blood was collected from the neck vessels. A small portion of the liver was immediately removed and fixed in 10 percent formalin solution and processed by dehydration in graded-strength ethanol, cleared in xylol, and embedded in paraffin. The paraffin sections of the liver tissue were stained with hematoxylin and eosin, and hepatotoxicity was evaluated by light microscopy. The major portion of the liver was quickly perfused in situ via the portal vein with ice-cold physiological saline, quickly excised, weighed, and used for biochemical experiments. All subsequent steps were performed at 2° to 4°C. The liver was homogenized in three volumes of 1.15 percent KCl with Potter-type glass homogenizer with Teflon pestle. The homogenates were centrifuged at 10,000 x g for 20 min followed by ultracentrifugation of the 10,000 x g supernatants at 105,000 x g for 60 min using the Beckman L5-50 ultracentrifuge. The pellets harvested by this procedure were used as microsomal fractions. Hepatic microsomal cytochrome P450 and b_5 contents were measured by the method of Omura and Sato (1964) using Shimazu MP5000 recording spectrophotometer. Protein concentration was determined according to Lowry et al. (1951). Hepatic injury was also assessed by estimating the activity of serum glutamic oxaloacetic transaminase.

Histological grading of the hepatic lesions was performed by the pathologist, who was not informed of the treatment received by the rats.

The student t-test was used to determine statistical significance of the difference among the various groups.

Results

Under conditions of hypoxia and chronic ethanol feeding, halothane anesthesia produced extensive hepatic necrosis (figure 1). The livers of animals that had been fed ethanol for 6 weeks and subsequently received halothane with low oxygen (10 percent) showed numerous areas of hepatic necrosis, primarily in pericentral areas of the lobules. These necrotic areas were frequently infiltrated with clusters of lymphocytes and neutrophils. The acidophilic bodies were often seen. Several hepatic cells in the centrilobular areas were swollen (vacuolated with fat droplets) and were more eosinophilic than normal. It should be noted that halothane anesthesia with ambient oxygen (35 percent) also resulted in hepatic damage when the rats had been fed ethanol chronically. The damage was apparent in most of these animals, but hepatic necrosis was milder in degree and extent than that in the animals treated with low oxygen concentration. Histologic study of the livers revealed only minimal changes in pair-fed animals that had received 10 percent oxygen without halothane, although mild fat droplets were seen in the livers of rats fed ethanol. Moreover, halothane anesthesia with 10 percent oxygen in pair-fed control rats showed no significant changes in hepatic morphology.

Hepatic microsomal cytochrome P450 content was increased by 30 percent after ethanol administration (table 1). However, the cytochrome P450 content of ethanol-fed rats was significantly decreased after halothane anesthesia with 10 percent oxygen. The cytochrome P450 content in the rat livers was not significantly changed after halothane anesthesia with 35 percent oxygen. Microsomal cytochrome b_5 was found to be unaltered by halothane anesthesia in ethanol-fed rats whether or not they had received adequate oxygen.

Levels of S-GOT increased significantly in ethanol-fed animals that had received halothane irrespective of oxygen concentration, but S-GOT values were much higher under hypoxic conditions (figure 2).

Discussion

Although the toxicity of halothane to the liver has been the subject of much interest and considerable controversy during the past decades (Brown et al. 1979), a consensus has been reached that it is a distinct but rare syndrome (Dienstag 1980). Since the earlier studies (Klatskin and Kimberg 1969; Paronetto and Popper 1970) on the pathogenesis of halothane hepatitis, investigators have attempted to demonstrate cell-mediated immune responses to halothane in patients with halothane hepatitis and suggested that halothane could produce hepatic injury by a hypersensitivity mechanism, possibly through halothane or one of its metabolites as a hapten. Although several studies (Mathieu et al. 1975; Reeves and McCracken 1976; Rosenberg and Wahlstorm 1973) have shown skin hypersensitivity or in vitro antibody formation to halothane or its metabolites, they failed to demonstrate hepatic damage.

More recently, there is evidence that halothane may be a direct hepatotoxin, and animal models of halothane hepatotoxicity have been described (McLain et al. 1979; Sipes and Brown 1976). McLain et al. (1979) have demonstrated that halothane hepatotoxicity can be produced by pretreatment of rats with microsomal enzyme inducers, such as phenobarbital, in combination with hypoxic condition. The hepatic lesions in these animals mainly consist of centrilobular necrosis often associated with infiltration of clusters of lymphocytes and neutrophils. Furthermore, it has been suggested that halothane might be metabolized to hepatotoxic intermediates by a reductive or non-oxygen-dependent cytochrome P450 dependent pathway in hepatic microsomes (McLain et al. 1979).

Because chronic ethanol administration has been shown to produce a proliferation of hepatic smooth endoplasmic reticulum associated with an enhancement of a variety of microsomal drug metabolizing enzymes, in both humans and rats (Ishii et al. 1973; Rubin and Lieber 1968), we wondered if halothane anesthesia affects the livers of rats fed ethanol chronically. It was found that halothane anesthesia produced extensive hepatic necrosis, primarily in pericentral areas of the lobules under conditions of hypoxia and chronic ethanol feeding. This histological finding was similar to those reported previously by using phenobarbital and hypoxic condition (McLain et al. 1979; Ross et al. 1979). Moreover, in the present study, hepatic necrosis was demonstrated also in rats fed ethanol chronically and subsequently anesthetized with halothane and oxygen (35 percent). Because it has been suggested (Israel et al. 1977) that the livers of ethanol-treated animals consume oxygen at high rates and are very sensitive to a reduction in the availability of oxygen, it is not unexpected that hepatic necrosis may occur in rats fed ethanol and anesthetized with halothane even at ambient oxygen concentrations. This finding is of interest in view of the recent report (Wood et al. 1980) that hepatic necrosis developed in rats pretreated with triiodthyronine and then anesthetized with halothane at an ambient oxygen concentration. They suggested that excess triiodthyronine induced an intracellular hypoxia, causing halothane to undergo reductive biotransformations to reactive metabolites, resulting in hepatic damage.

Although precise mechanisms by which halothane produces hepatic necrosis in these animals are unknown, Uehleke et al. (1973) have previously shown in vitro that with reduced oxygen concentrations, halothane produces far more covalent binding to hepatic constituents than in the presence of excess oxygen. Moreover, Widger et al. (1976) substantiated that increased covalent binding enhanced inorganic fluoride release and histologic damage with hypoxic oxygen concentrations during halothane anesthesia. These data suggest that altered halothane metabolism to a reductive reaction could lead to reactive metabolites interacting with hepatic cells or its organelles, resulting in hepatic necrosis. Therefore, it is concluded that our animal model may be predicated on decreased oxygen availability to already induced microsomal enzymes by ethanol so that reductive metabolism of halothane is activated.

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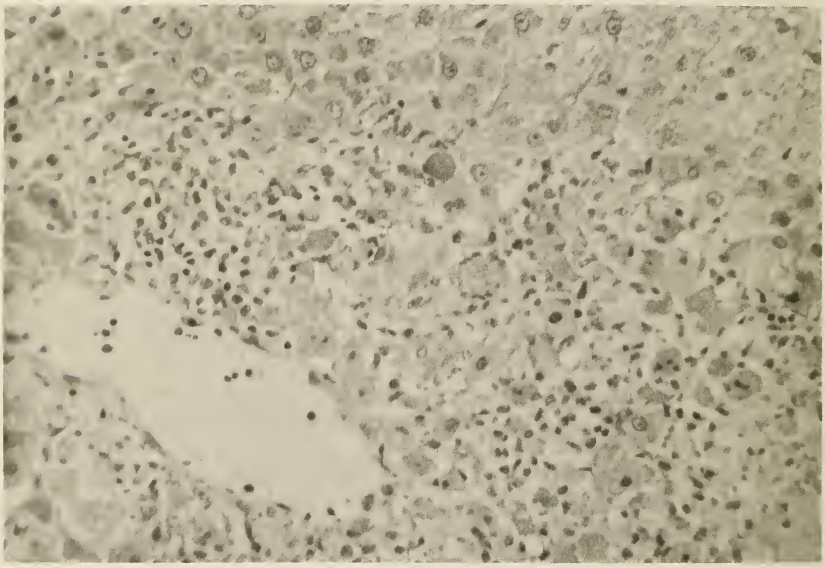
Figure 1. Hepatic Morphology 24 Hours after Halothane 1.0 Percent; at Low Oxygen Concentration (10 Percent) in an Ethanol-Treated Rat



Centrilobular necrosis with leukocyte infiltration and eosinophilic bodies. (HE X200)

Figure 2. Changes of Serum Transaminase Activities after Halothane Anesthesia in Ethanol-Treated Rat

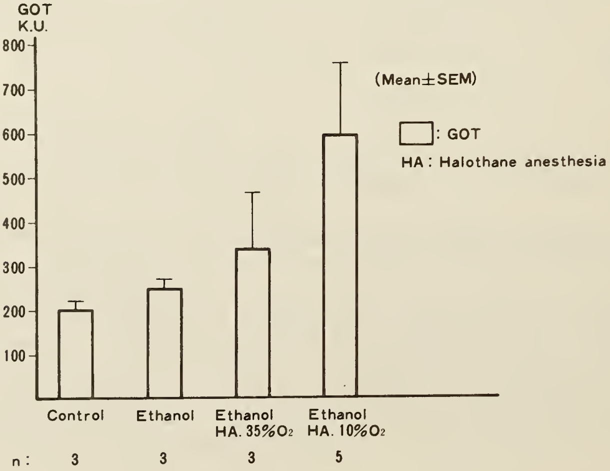


Table 1. Effect of Halothane Anesthesia on Hepatic Microsomal Cytochrome P450 and b_5 Contents in Ethanol-fed Rats

Treatment	Cytochrome P450 (n mole/mg protein)	Cytochrome b_5 (n mole/mg protein)
Control (n=3)	0.58 ± 0.11	0.35 ± 0.04
Ethanol (n=3)	0.75 ± 0.02*	0.36 ± 0.02
Ethanol (n=3) Halothane, 35 percent O_2	0.78 ± 0.10	0.40 ± 0.04
Ethanol (n=5) Halothane, 10 percent O_2	0.55 ± 0.06**	0.44 ± 0.03

* vs.
** $p < 0.05$.
Note: Data express Mean ± SEM.

Enhancement of Acute Drug Hepatitis by Chronic Ethanol: Onset, Progression, and Resolution

Jean-Gil Joly and Claude Hétu

The acute liver toxicity of several xenobiotics has been related to their biotransformation to highly reactive metabolites via cytochrome P-450-dependent mixed function oxidases, and to covalent binding of these metabolites to cellular macromolecules. Studies using acetaminophen and bromobenzene have shown the importance of factors leading to an imbalance between the rate of production of reactive metabolites and the rate of their disposition through detoxification pathways in determining the occurrence and severity of drug-induced liver lesions (Gillette 1974*a*,*b*; Mitchell and Jollow 1975).

The induction of cytochrome P-450-dependent mixed function oxidases is known to have a marked influence on the hepatotoxicity of xenobiotics susceptible to metabolic activation through this pathway. Depending on the type of inducer administered, and therefore on the catalytic properties of the cytochrome P-450 form(s) induced by a particular agent, an increase or a decrease in the rate of production of toxic metabolites has been correlated with an enhancement, or a reduction, in covalent binding and severity of liver lesions (Gillette 1974*b*; Mitchell and Jollow 1975).

Most information on this aspect of drug interactions results from studies using phenobarbital and 3-methylcholanthrene as inducers. Ethanol, however, is a widely consumed inducer of liver mixed function oxidases (Rubin et al. 1968). Moreover, we have shown (Villeneuve et al. 1976) that ethanol increases the liver content in form(s) of cytochrome P-450 catalytically distinct from those induced by other agents. Whether chronic ethanol consumption can result in the enhancement, through this or other mechanisms, of the hepatotoxic effects of xenobiotics may thus be of considerable importance from a clinical and epidemiological standpoint.

We have examined and compared, in the present study, the effects of chronic ethanol administration in the rat on the severity of hepatitis produced by three chemically distinct types of xenobiotics: acetaminophen, bromobenzene, and dimethylnitrosamine. Our results provide evidence that, although ethanol enhances the hepatotoxicity

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NOTE: Figures and tables appear at end of paper.

of these compounds, this effect cannot be explained simply by a more rapid and more severe onset of hepatitis associated with increased cytochrome P-450-dependent metabolic activation alone, it also results from other effects of chronic ethanol consumption on the liver.

Experimental Procedures

Female Sprague-Dawley rats (120 to 150 g), pair-fed for 21 days with De Carli and Lieber's (1967) liquid diet containing ethanol (36 percent of total calories), isocalorically replaced by carbohydrates in controls, were used in all experiments. Animals were fasted for 16 hours prior to administration of acetaminophen (1 g/kg, by gastric gavage), bromobenzene (500 mg/kg, intraperitoneally, as a 125 mg/ml solution in corn oil), or dimethylnitrosamine (50 mg/kg, intraperitoneally, as a 10 mg/ml solution in saline). Ethanol pretreated and control animals were then given the control liquid diet for the remaining duration of the experimental period.

Serum enzyme markers of liver necrosis, glutamate pyruvate transaminase (GPT) and glutamate oxalacetate transaminase (GOT), were assayed with "Opt. UV-system A" kits from Boehringer Mannhein. Whole liver homogenates were used for determination of liver cytochrome P-450 content (Joly et al. 1975), glutathione (reduced form) content (Mitchell et al. 1973), and in vivo covalent binding of ^{3}H-acetaminophen to liver proteins (Jollow et al. 1973). The rate of formation of the glutathione conjugate of acetaminophen by liver microsomes in vitro was determined according to Buckpitt et al. (1977). The severity of liver lesions produced by the three hepatotoxins was arbitrarily graded on a 0 to 4+ scale by double blind histological evaluation (Dixon et al. 1975).

Results and Discussion

The effect of chronic ethanol administration on the liver toxicity of acetaminophen was first examined. At the given dose, mortality after acetaminophen was encountered only in animals pretreated with ethanol: 3 of 13 animals were dead at 36 hours, and 11 out of 18 died within 60 hours. As seen in figure 1, acetaminophen administration was associated with an earlier significant rise in serum GPT level in ethanol-pretreated rats than in controls. Peak GPT level in ethanol-fed rats was much higher than in controls, occurred at a time when values had returned to almost normal in the latter, and remained significantly elevated 60 hours after acetaminophen administration. Similar results were obtained using glutamate oxalacetate transaminase as serum enzyme marker of liver necrosis.

As seen in figure 2, assessment of the severity of the hepatitis produced by acetaminophen in these animals reveals the existence of a close correlation, in time as well as in relative intensity, between histological and biochemical indices of liver lesions. Centrolobular liver necrosis occurred earlier in ethanol-pretreated animals and was much more severe and lasted much longer than in controls. In ethanol-fed rats, 60 hours after acetaminophen the histological picture of hepatitis was severe and dissociated from the blood levels of the serum markers of cytolysis.

Initial depletion of liver glutathione content after acetaminophen administration was found to proceed at a rate 45 percent higher in ethanol-pretreated rats than in controls, and was associated (table 1) with a sixfold increase over controls in the amount of ^{3}H-acetaminophen-derived radioactivity covalently bound to liver protein 4 hours after administration of the drug.

This increase in covalent binding in vivo was related with the induction of liver microsomal mixed function oxidases by chronic ethanol administration, as reflected by higher liver cytochrome P-450 content (72 versus 46 nmol/g liver) in ethanol-fed rats than in controls, and with the associated threefold increase in the rate of microsomal production of reactive metabolites of acetaminophen conjugating with glutathione in vitro (table 1).

Chronic ethanol pretreatment was thus found to increase the severity of the progression and evolution of hepatitis following acetaminophen administration. The above data also suggested that this effect of ethanol could be explained mainly by an increase in the importance of the resulting liver necrosis, secondary to the induction of liver microsomal mixed function oxidases by chronic ethanol administration.

Such an explanation, moreover, suggested that induction of liver mixed function oxidases by chronic ethanol administration could also be responsible for an increase in the severity of liver lesions produced by other substances known to undergo metabolic activation to hepatotoxic derivatives. This eventuality was thus examined using bromobenzene, a model substance whose mechanism of hepatotoxic action has been well characterized (Gillette 1974*a*,*b*).

As shown in table 2, chronic ethanol pretreatment resulted in a 2.7-fold increase in the lethality of the given dose of bromobenzene in the rat for the time period studied (36 hours after bromobenzene administration). It also strongly enhances the severity of bromobenzene-induced hepatitis, as shown by much larger elevations in biochemical and histological indices of liver necrosis in ethanol-pretreated rats. In the latter, a twofold increase in the rate of depletion of liver glutathione, in the initial period following administration of bromobenzene, again suggested that the rate of production of toxic bromobenzene-3,4-epoxide (Gillette 1974*b*) was markedly accelerated in these animals, thus accounting for the increased severity of liver lesions produced by bromobenzene in ethanol-fed rats.

Studies with these two hepatotoxins had thus shown that the induction of liver mixed function oxidases by chronic ethanol administration could play a major role in the enhancement, by ethanol, of acute drug hepatitis. The observed persistence of histological signs of severe acetaminophen-induced hepatitis in ethanol-pretreated rats, at a time when healing processes were well engaged in controls, however, suggested that additional mechanism(s) also could be responsible for the effect of ethanol on xenobiotic hepatotoxicity.

This possibility was strengthened by studies (Duguay et al. 1979) showing that chronic ethanol administration causes a delayed and decreased liver regeneration activity, as assessed by three different criteria, following partial hepatectomy. This effect, which did not require the actual presence of ethanol in the animal circulation, was also found to persist for at least 3 days following ethanol withdrawal and to have subsided after a 7-day abstinence period. That this inhibitory influence of ethanol is probably not related to its action as an inducer of liver mixed function oxidases was also suggested by the observation of normal regeneration activity in rats pretreated with phenobarbital.

The participation of this inhibitory effect of ethanol on hepatocyte replication in its enhancing influence on drug hepatitis is difficult to evaluate. Preliminary studies, however, suggest that its importance may be determinant in the evolution and outcome of dimethylnitrosamine-induced hepatitis in rats pretreated with ethanol.

As shown in figures 3 and 4, the administration of dimethylnitrosamine in ethanol-pretreated and control rats is followed, during the first 48 hours, with similar rises in serum GPT levels, parallel decreases in liver microsomal protein and cytochrome P-450 content (data not shown), and similar rises in histological indices of liver necrosis in both groups. Chronic ethanol pretreatment, in this case, did not appear to increase the severity of the onset and progression of liver lesions caused by this hepatotoxin up to 48 hours after DMN administration.

Ethanol pretreatment, however, was found to have a profound influence on the resolution of dimethylnitrosamine-induced hepatitis. Thus, the administered dose of this hepatotoxin was found, in a group of 10 pairs of animals kept for up to 4 days, to be lethal for all ethanol-pretreated rats, whereas all controls survived and were clearly recovering. This difference was also associated, at the end of the period, with marked differences in liver histology between the two groups. In the livers of control animals, the necrotic processes were arrested, with necrotic zones being cleared; numerous mitotic figures were also present, indicating that regeneration activity was well underway at that time. By contrast, histological examination of livers from ethanol-pretreated rats showed that at that time of death, between 2 and 4 days after dimethylnitrosamine administration, centrolobular hemorrhagic necrosis was persisting, whereas no mitotic figures could be detected. Hypoglycemia was ruled out as a cause of lethality following measurement of blood glucose.

Ethanol pretreatment, in this case, appears to exert a marked influence mostly on the recovery from liver lesions produced by dimethylnitrosamine. Whether ethanol pretreatment influences repair or regeneration processes remains to be determined by direct means.

Conclusion

Chronic ethanol administration in the rat clearly enhances the severity of acute hepatitis resulting from the administration of certain xenobiotics. This effect of ethanol can be explained, at least in part, by the induction of cytochrome P-450-dependent liver mixed function oxidases associated with chronic ethanol administration. An acceleration in the rate of production of toxic reactive metabolites can thus account for increases, in some cases, in the initial severity of liver lesions produced in ethanol-pretreated animals.

Such an explanation, however, cannot account for all the observed effects of chronic ethanol administration on drug-induced hepatitis, as suggested in these studies. Interferences with other mechanisms coming into play late (48 hours) after the onset of the hepatitis, possibly related to cellular repair and/or regeneration processes, also appear to be a significant participating factor in the progression and resolution of acute drug hepatitis. This effect of ethanol, which can be observed in the liver after surgical trauma, may in some cases play a determinant role in recovery from drug-induced liver disease.

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Figure 1. **Serum Glutamate Pyruvate Transaminase Following Oral Administration of Acetaminophen (1 g/kg) in Rats Chronically Fed an Ethanol-Containing Liquid Diet and Their Controls ($\overline{X}$ ±S.F.)**

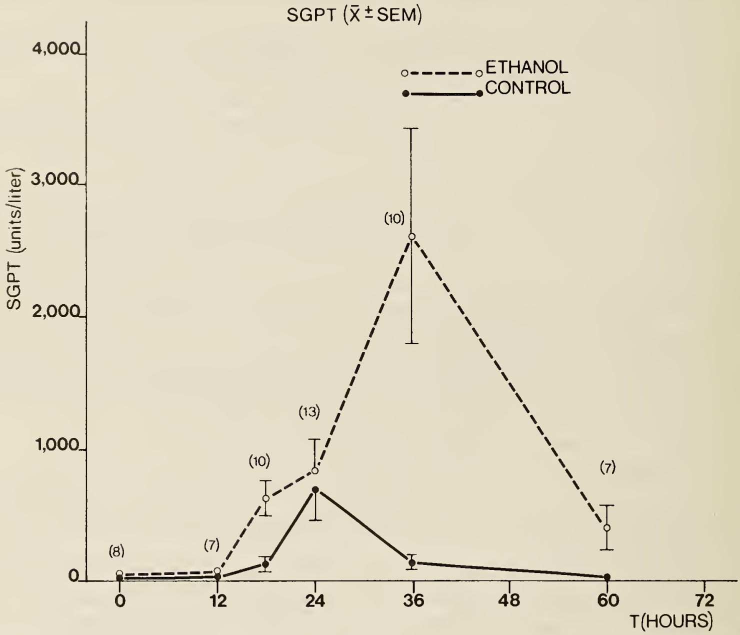

Figure 2. **Severity of Liver Lesions, by Histological Assessment, Following Oral Administration of Acetaminophen (1 g/kg) in Rats Chronically Fed an Ethanol-Containing Liquid Diet and Their Controls**

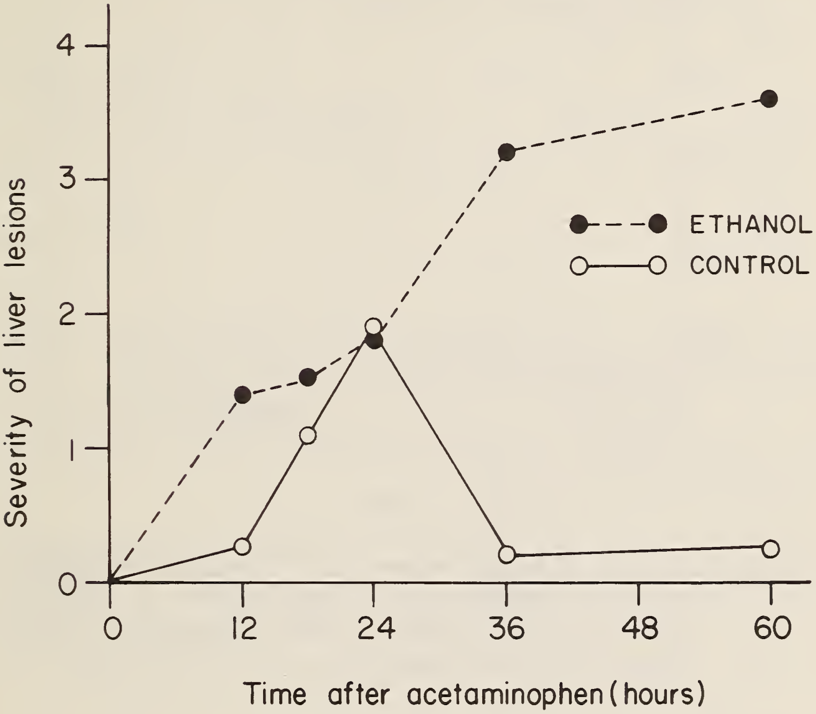

Figure 3. Serum Glutamate Pyruvate Transaminase Following Intraperitoneal Administration of Dimethylnitrosamine (50 mg/kg) in Rats Chronically Fed an Ethanol-Containing Liquid Diet and Their Controls ($\overline{X} \pm$ S.E.)

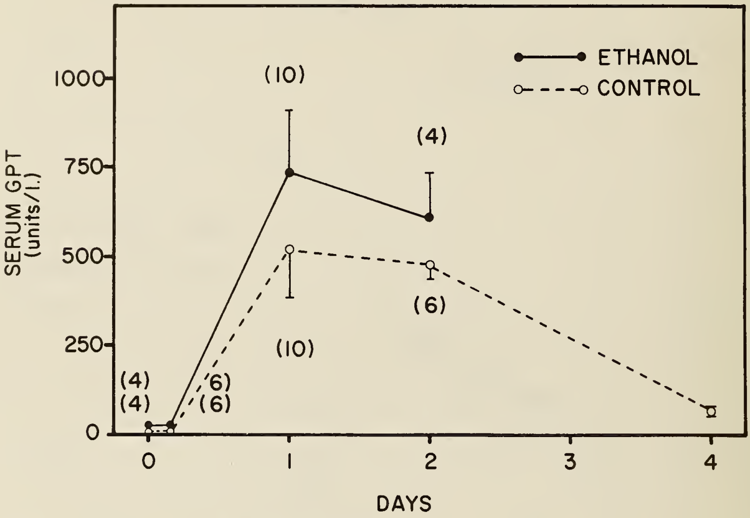

Figure 4. Severity of Liver Lesions, by Histological Assessment, Following Intraperitoneal Administration of Dimethylnitrosamine (50 mg/kg) in Rats Chronically Fed an Ethanol-Containing Liquid Diet and Their Controls

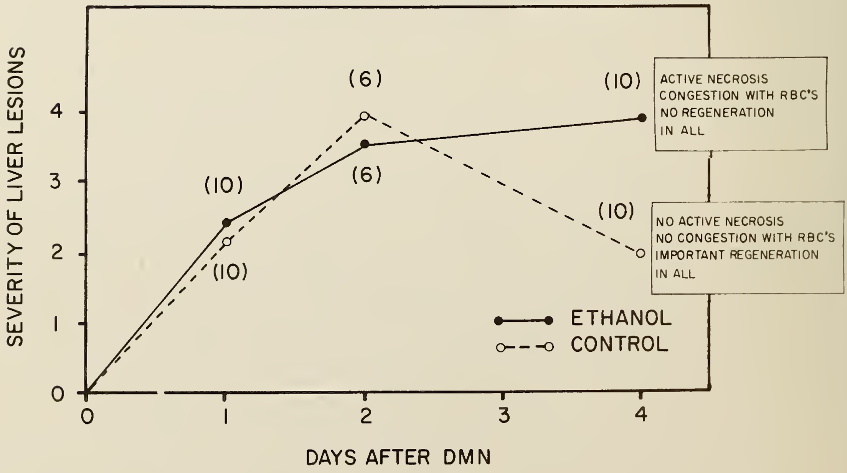

Table 1. Effect of Chronic Ethanol Pretreatment on Microsomal Production of Acetaminophen-Glutathione Conjugate (AAP-S-G) In Vitro and on Covalent Binding of Acetaminophen to Liver Proteins In Vivo

	Pretreatment	
	Control	Ethanol
AAP-S-G (nmole/min/mg microsomal protein)	0.38 ± 0.02	1.11 ± 0.16*
AAP covalent binding (nmole/mg liver protein)	0.10 ± 0.01	0.58 ± 0.02**

*$p < 0.005$.
**$p < 0.001$.

Table 2. Effect of Chronic Ethanol Pretreatment on Indices of the Severity of Liver Lesions 36 Hours after Bromobenzene Administration

	Pretreatment	
	Control	Ethanol
Serum enzymes (u/ℓ)		
GPT	506 ± 240	6142 ± 1141*
GOT	861 ± 306	7433 ± 1411*
Histological index of liver necrosis	1+	3+
Mortality	3/18	8/18

*$p < 0.001$.

Effect of Chronic Alcohol Pretreatment on the Hepatotoxicity Elicited by Chlorpromazine, Paracetamol, and Dimethylnitrosamine

Rolf Teschke

Introduction

Ethanol is oxidized to acetaldehyde in the liver by cytosolic alcohol dehydrogenase (ADH) (Teschke et al. 1976) and the microsomal ethanol oxidizing system (MEOS) (Lieber and DeCarli 1970, 1972; Moreno et al. 1979, in press; Petrides et al. in press; Teschke 1980; Teschke and Lieber 1980; Teschke et al. 1972, 1974, 1976), whereas catalase localized in the hepatic peroxisomes appears to play no significant role (Teschke et al. 1976).

The constituents of MEOS include cytochrome P-450, NADPH-cytochrome *c* reductase, and phospholipids (Lieber et al. 1978; Miwa et al. 1978; Ohnishi and Lieber 1977), all of which are required also for the activity of various other microsomal enzymes metabolizing drugs or other exogenous compounds (Lu et al. 1973). Ethanol and other exogenous compounds may therefore compete for the common metabolic site at the cytochrome P-450 level (figure 1). These characteristics are the biochemical basis for the inhibition of drug metabolism (Rubin et al. 1970), as well as for the decreased hepatotoxicity elicited by carbon tetrachloride (Teschke, Hauptmeier, Minzlaff, Frenzel, and Strohmeyer 1980) or paracetamol (Sato et al. in this volume) observed following an acute dose of ethanol.

This paper will focus on the effect of chronic pretreatment with alcohol on the hepatotoxicity produced by various exogenous compounds such as chlorpromazine, paracetamol, and dimethylnitrosamine. Prolonged ethanol intake leads to an induction of microsomal enzyme activities (Joly et al. 1973; Rubin and Lieber 1968), a condition which may alter the effect of potentially hepatotoxic agents. An increased susceptibility after chronic alcohol consumption has previously been demonstrated for carbon tetrachloride (Hasumura et al. 1974).

NOTE: Figures and table appear at end of paper.

Alcohol and Chlorpromazine

Psychotropic drugs, including phenothiazines, are widely used and may cause cholestasis due to microsomal metabolism of the parent compound to toxic intermediates (Samuels and Carey 1978). Since the use of phenothiazine derivatives such as chlorpromazine is not uncommon in connection with alcohol consumption, the question arose whether chronic alcohol consumption may predispose to chlorpromazine-induced cholestasis.

To study the effect of chronic alcohol intake on the hepatotoxicity due to an acute dose of chlorpromazine, rats were pair-fed for 4 weeks with nutritionally adequate liquid diets (DeCarli and Lieber 1967) containing either 36 percent of total calories as ethanol (alcohol diet) or isocaloric carbohydrates (control diet). To test the effect of an acute drug administration after chronic ethanol consumption, rats fed either the ethanol-containing diet or the control diet were given the control diet during the 18 hours preceding the drug administration to allow complete clearing of ethanol from the blood. The diets were then replaced by tap water, and the animals (alcohol and control rats) received chlorpromazine at doses of 15, 30, or 45 mg/kg body weight (BW) dissolved in physiological saline solution by intraperitoneal injection. Some animals (alcohol and control rats) received physiological saline solution only. The animals were killed by decapitation 18 hours after drug administration. Blood was collected from the neck vessels and serum enzyme activities were determined (Teschke, Stutz, and Moreno 1980).

When compared to animals receiving the control liquid diet, prolonged alcohol administration for 4 weeks resulted in a significant rise of alkaline phosphatase (AP) activity in the serum under experimental conditions where no chlorpromazine was administered (figure 2). Moreover, the administration of chlorpromazine in increasing amounts up to 45 mg/kg BW failed to enhance serum AP activity in rats fed the control diet when compared to control animals receiving no chlorpromazine. Conversely, in rats chronically pretreated with alcohol chlorpromazine in increasing amounts up to 45 mg/kg BW there was a correspondent enhancement of serum AP activity. In particular, the serum AP activity observed in the alcohol animals following the largest dose of chlorpromazine (45 mg/kg BW) was significantly higher than in the alcohol-pretreated animals receiving no chlorpromazine (figure 2). Since increases of serum alkaline phosphatase activities are suggestive for cholestasis, it is concluded from these experiments that chronic alcohol pretreatment may predispose to chlorpromazine-induced cholestasis.

Compared to control animals, chronic alcohol consumption per se led to a significant increase of serum glutamate-pyruvate-transaminase (GPT) activity (figure 3), an enzyme confined to the cytosol of the hepatocyte. The administration of chlorpromazine in increasing amounts resulted in a steady rise of serum GPT activity that was somewhat more pronounced in the alcohol-pretreated animals than in the corresponding control rats (figure 3). Similar results were obtained for the serum activities of glutamate-oxalacetate-transaminase (GOT) (figure 4), an enzyme of cytosolic as well as mitochondrial origin of the hepatocyte.

In addition, chronic alcohol consumption led to slight increases of serum glutamate dehydrogenase (GDH) activities compared to animals fed the control diet (figure 5). After administration of chlorpromazine serum, GDH activity increased in both animal groups fed either the control or the alcohol diet. However, with the largest dose of chlorpromazine (45 mg/kg BW) the difference in GDH activity between the alcohol and control animals was much more striking than with lower doses of chlorpromazine (figure 5). Since GDH activity is primarily localized in liver mitochondria, these results suggest that chronic alcohol pretreatment predisposes not only to chlorpromazine-induced cholestasis as evidenced by increased activities of serum AP (figure 2), but also to chlorpromazine-mediated hepatocellular damage, as shown by increased serum GDH activities (figure 5).

These data, therefore, show that chronic alcohol consumption may favor the toxifying pathway (figure 6) of a chemical compound such as chlorpromazine, which may be metabolized more rapidly to its toxic intermediate(s).

The view is generally held that chlorpromazine is extensively metabolized by liver microsomes (Gillette and Kamm 1960). In this reaction, the methyl group is being hydroxylated (Coccia and Westerfeld 1967), and cytochrome P-450 may function as the terminal oxidase (Gigon and Bickel 1971). In addition, liver microsomes are capable of generating chlorpromazine free radicals from the parent compound chlorpromazine (Piette et al. 1964; Samuels and Carey 1978). The chlorpromazine free radicals may then react with oxygen to form stable chlorpromazine sulfoxide metabolites (Felmeister and Discher 1964; Samuels and Carey 1978). It has been postulated that the chlorpromazine free radical irreversibly inhibits a variety of enzymes, including Na^+, K^+-ATPase, via an interaction with the enzyme's free sulfhydryl groups, and this inhibition was partially prevented by reduced glutathione (Samuels and Carey 1978). Chlorpromazine or one of its metabolites may therefore conceivably induce cholestasis by a direct toxic effect on the bile secretory mechanism of the liver through an interaction with canalicular membrane ATPases.

Moreover, the degree of cholestasis may be influenced by the extent of microsomal conversion of chlorpromazine to more active metabolites in form of free radicals or to minimally active metabolites such as chlorpromazine sulfoxide (Samuels and Carey 1978).

Since chronic alcohol consumption leads to increased activities of various hepatic microsomal drug metabolizing enzymes (Joly et al. 1973; Rubin and Lieber 1968) and to an enhanced content of cytochrome P-450 (Hasumura et al. 1975; Rubin et al. 1968; Villeneuve et al. 1976), it is conceivable that under these experimental conditions chlorpromazine is metabolized more rapidly to toxic free radicals. The latter may in turn promote cholestasis, as evidenced by increased serum AP activities (figure 2), which is associated with increased hepatocellular damage as demonstrated by the enhanced activities of various serum enzymes originating from the liver (figures 3, 4, 5). In addition, prolonged treatment with alcohol has been reported to result in a decreased liver content of reduced glutathione (Shaw et al. 1979), which in turn could further promote the development of cholestasis due to a decreased capacity of the liver to scavenge the extensively formed chlorpromazine free radicals.

Alcohol and Paracetamol

It has been suggested that due to microsomal enzyme induction, alcoholics may also be more susceptible to commonly prescribed drugs such as paracetamol (Barker et al. 1977), which is metabolized to toxic metabolites via the cytochrome P-450-dependent mixed function oxidase (Mitchell and Jollow 1975). In the present experiments, the question was studied whether under controlled experimental conditions an increased paracetamol-induced hepatotoxicity could be observed following chronic alcohol consumption (Teschke et al. 1979). The experimental conditions were the same as in the study with chlorpromazine described above, except that paracetamol instead of chlorpromazine was given acutely.

Compared to results on animals receiving the control liquid diet, chronic alcohol consumption results in a slight increase of serum glutamate-oxalacetate-transaminase (GOT) activities under experimental conditions where no paracetamol was administered (figure 7). Moreover, the application of paracetamol to rats pretreated with the control diet for 4 weeks led to a slight increase of GOT activity when the drug was given in increasing amounts up to 800 mg/kg BW (figure 7). Similar results were obtained in the alcohol-pretreated animals receiving the same acute dose of paracetamol (figure 7). Conversely, a striking enhancement of serum GOT activity could be demonstrated following a high dose of paracetamol (1200 mg/kg BW), and this increase was much more pronounced in the alcohol-fed animals compared to their pair-fed littermates receiving the control diet (figure 7).

Since GOT originates from hepatic mitochondria and/or hepatic cytoplasm, the activity of other enzymes was determined to assess the localization of the hepatocellular damage due to the administration of paracetamol. Therefore, the activity of glutamate dehydrogenase was determined an enzyme exclusively confined to hepatic mitochondria. Compared to controls, rats fed the alcohol-containing diet for 4 weeks exhibited a slight increase of GDH activity in the serum, whether or not a single low dose of paracetamol (400 mg/kg BW) or physiological saline solution was injected (figure 8). A striking enhancement of serum GDH activity could be observed, however, when intermediate or high doses of paracetamol (800 or 1,200 mg/kg BW) were administered (figure 8). In accordance with the data obtained for GOT (figure 7), the increase of GDH activities after 800 or 1,200 mg paracetamol/kg BW was much more striking in the alcohol-pretreated rats than in their pair-fed controls receiving the same amount of paracetamol (figure 8). Further studies with high doses of paracetamol failed to show a similar increase of activity for cytoplasmic GPT (figure 9), indicating that hepatocellular injury due to paracetamol can be confined primarily to hepatic mitochondria.

It is generally accepted that paracetamol is excreted to a major extent as sulfate or glucuronide (Mitchell and Jollow 1975). Paracetamol metabolism, however, also proceeds via the cytochrome P-450-dependent microsomal mixed function oxidase, which may result in the formation of toxic intermediates. Glutathione normally combines with the metabolite and forms a readily excreted mercapturic acid, preventing a combination of the toxic intermediate with liver macromolecules essential for a regular function of the hepatocyte (Mitchell and Jollow 1975). Since chronic alcohol consumption results in an induction of various microsomal enzyme activities including the mixed function oxidase consisting of cytochrome P-450 and NADPH-cytochrome P-450 reductase (Hasumura et al. 1975; Joly et al. 1973; Rubin et al. 1968), it is conceivable that the metabolism of paracetamol increases following chronic alcohol consumption like it does with other drugs (figure 10). This will result in an increased formation of hepatotoxic metabolites, which in turn may lead to an increased paracetamol-induced hepatotoxicity following chronic alcohol consumption due to depletion of glutathione. Thus, the clinical observation of an increased paracetamol-induced hepatotoxicity following chronic alcohol consumption (Barker et al. 1977) could be substantiated in the present study under controlled experimental conditions in accordance with other reports (Sato et al. in this volume). Like for chlorpromazine (figures 2 through 5), chronic alcohol consumption also favors the toxifying pathway (figure 6) for paracetamol. In contrast to the data obtained with chlorpromazine (figure 2), however, chronic alcohol consumption failed to predispose to cholestasis, since serum activities of alkaline phosphatase remained virtually unchanged at higher doses of paracetamol following chronic pretreatment with alcohol (figure 11).

Alcohol and Dimethylnitrosamine

Previous studies have shown that the administration of phenobarbital as a classical inducer for microsomal enzymes leads to a reduction of the hepatotoxicity elicited by nitrosamines (Nayak et al. 1975). The question to be studied was whether similar data can be obtained following chronic ethanol intake, which also leads to microsomal enzyme induction. Rats were therefore pair-fed liquid diets containing either ethanol (36 percent of total calories) or isocaloric carbohydrates as controls for 23 days. Subsequently, the animals received on 5 consecutive days either dimethylnitrosamine (DMN; 1 mg/100 g BW, dissolved in physiological saline; 1 mg/1 ml) or saline intraperitoneally in equal amounts. The animals were killed 24 hours after the last application (Gellert et al. 1980; Teschke, Gellert, Haydn, Frenzel, Oldiges, and Strohmeyer 1980).

Chronic pretreatment with the liquid control diet before the intraperitoneal administration of dimethylnitrosamine (DMN) on 5 consecutive days resulted 24 hours after the last application in a significant rise of serum glutamate dehydrogenase (GDH), glutamate-oxalacetate-transaminase (GOT), and glutamate-pyruvate-transaminase (GPT) activities when compared to animals fed the same liquid control diet receiving physiological saline intraperitoneally instead of DMN (table 1). There was a striking increase of GDH activity by 350 percent ($p<0.0025$), of GOT activity by 45 percent ($p<0.05$), and of GPT activity by 120 percent ($p<0.0025$), indicating severe hepatic injury due to DMN by biochemical evaluation. These animals showed massive hepatic centrolobular necrosis following DMN applications (figure 12). The necrotic area was sharply demarcated from intact liver parenchyma. In addition, hydropic degeneration, Councilman bodies, focal fibrosis, and inflammatory infiltrations of the periportal regions could be observed in all animals under these experimental conditions (figure 12). Conversely, animals fed the control diet receiving only saline intraperitoneally instead of DMN showed a normal liver cell architecture.

The striking increase of GDH, GOT, and GPT activities in the serum following DMN administration in animals fed the control diet (table 1) was almost completely prevented after ethanol pretreatment of the rats. Animals receiving DMN on 5 consecutive days following chronic alcohol pretreatment for 23 days showed virtually no change of serum GDH and GOT activity compared to rats pretreated with the alcohol diet receiving saline instead of DMN. Only GPT activity was elevated by 55 percent ($p<0.0025$) under these experimental conditions (table 1). Histological assessment revealed only few single-cell necrosis, few Councilman bodies, swollen liver cells in the periphery of the hepatic lobule, and some cells with fatty degeneration (figure 13). Thus, the livers of alcohol-pretreated animals (figure 13) showed many fewer signs of hepatic injury after the same amount of DMN when compared with those of animals fed the control diet (figure 12), findings confirmed by biochemical assessment (table 1).

There is considerable dispute concerning the metabolic pathway of DMN in the liver cell. Oxidative demethylation has been suggested to be one of the activating steps in DMN metabolism (Cottrell et al. 1977), partly mediated via a cytochrome P-450-dependent enzyme system (Czygan et al. 1973) involving various forms of cytochrome P-450 (Lotlikar et al. 1975). However, other studies have suggested that the metabolism of DMN may also proceed via a noncytochrome P-450-dependent pathway (e.g., Lake et al. 1977). Several other forms of DMN-demethylases have also been incriminated in the metabolism of DMN (Kroeger-Koepke and Michejda 1979; Seitz et al. 1979). The exact mechanism of DMN metabolism in the liver therefore remains to be established.

The present data show that chronic alcohol consumption protects from hepatotoxicity due to DMN, most probably as a consequence of an enhancement of detoxifying rather than toxifying pathways of the parent compound or its toxic metabolites (figure 6). A similar observation has been made following pretreatment with the microsomal inducer phenobarbital (Kunz et al. 1969; Phillips et al. 1977). Of particular interest is the finding that pretreatment with either phenobarbital (Nayak et al. 1975) or alcohol (Schmähl et al. 1965) also reduces the incidence of liver carcinomas due to nitrosamines by 26 percent and 20 percent, respectively. With regard to alcohol, similar data were obtained in preliminary studies by our own group.

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Figure 1. Microsomal Components Required for the Hepatic Metabolism of Ethanol, Drugs, Carcinogens, and Procarcinogens

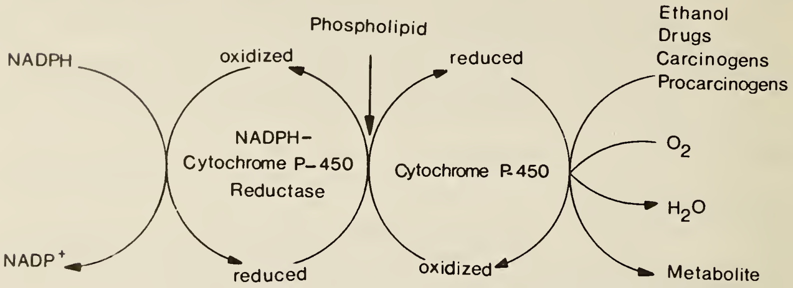
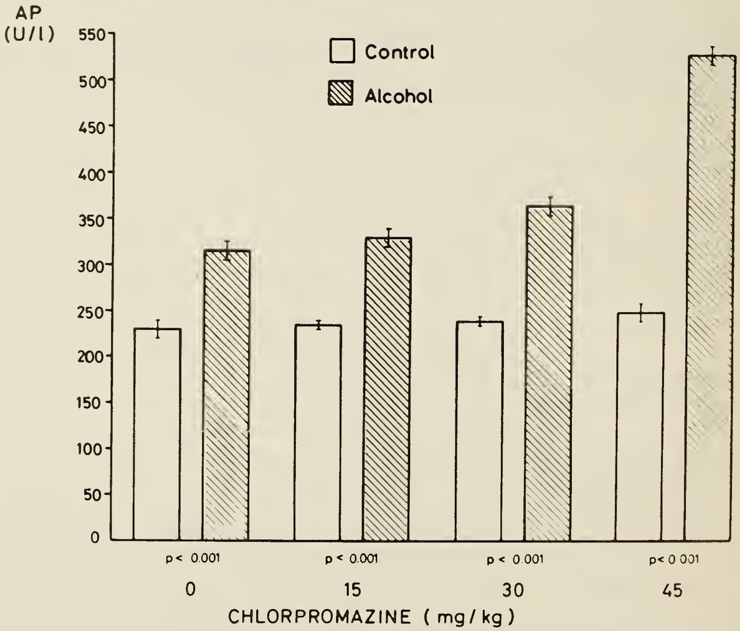
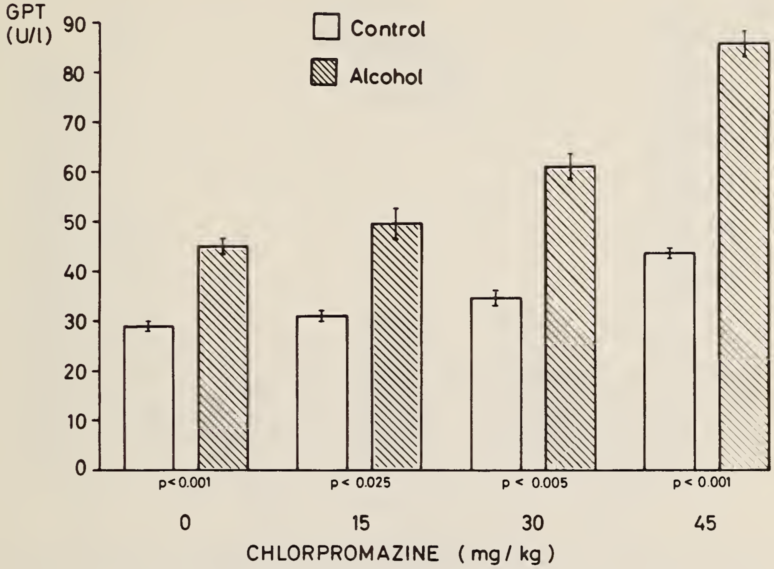


Figure 2. Effect of an Acute Dose of Chlorpromazine on Serum Alkaline Phosphatase (AP) Activity Following Chronic Alcohol Consumption



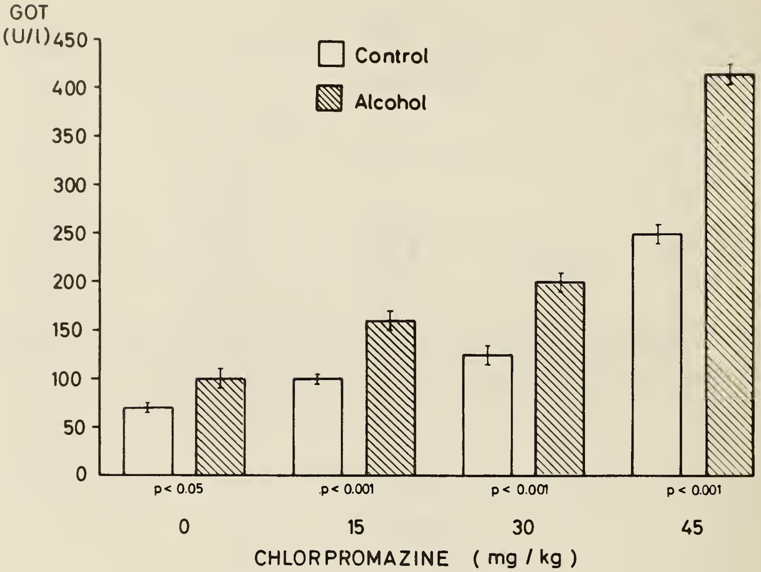
Rats were pair-fed for 4 weeks diets containing ethanol (36 percent of total calories), whereas the control diet contained additional carbohydrates instead of ethanol. Eighteen hours after ethanol withdrawal, chlorpromazine was administered intraperitoneally in doses as indicated, and serum AP activity was determined 18 hours thereafter. Each experimental group consisted of six animals. (From Teschke, R.; Stutz, G.; Moreno, F. Cholestasis following chronic alcohol consumption: Enhancement after an acute dose of chlorpromazine. *Biochem Biophys Res Commun*, 94:1013-1020, 1980. Copyright 1980 by Academic Press Inc. [NY].)

Figure 3. Effect of an Acute Dose of Chlorpromazine on Serum Glutamate-Pyruvate-Transaminase (GPT) Activity Following Chronic Alcohol Consumption



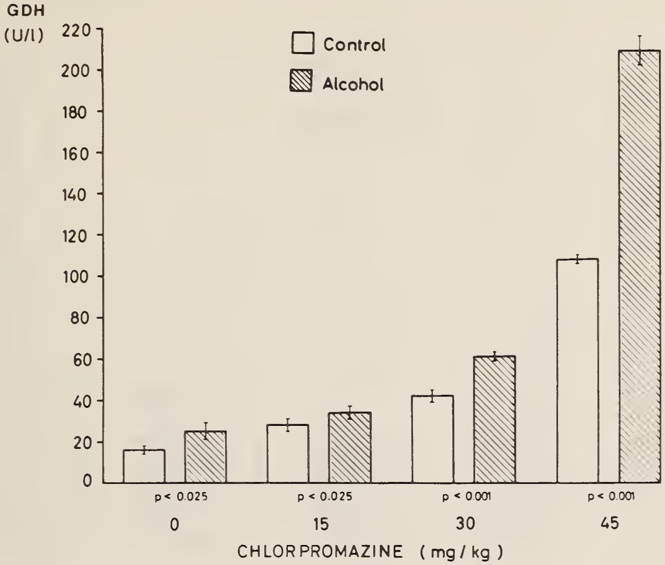
The experimental conditions are given in the legend for figure 2. (From Teschke, R.; Stutz, G.; and Moreno, F. Cholestasis following chronic alcohol consumption: Enhancement after an acute dose of chlorpromazine. *Biochem Biophys Res Commun,* 94:1013-1020, 1980. Copyright 1980 by Academic Press Inc. [NY].)

Figure 4. **Effect of an Acute Dose of Chlorpromazine on Serum Glutamate-Oxalacetate-Transaminase (GOT) Activity Following Chronic Alcohol Consumption**



The experimental conditions are given in the legend for figure 2. (From Teschke, R.; Stutz, G.; and Moreno, F. Cholestasis following chronic alcohol consumption: Enhancement after an acute dose of chlorpromazine. *Biochem Biophys Res Commun,* 94:1013-1020, 1980. Copyright 1980 by Academic Press Inc. [NY].)

Figure 5. Effect of an Acute Dose of Chlorpromazine on Serum Glutamate Dehydrogenase (GDH) Activity Following Chronic Alcohol Consumption



The experimental conditions are given in the legend for figure 2. (From Teschke, R.; Stutz, G.; and Moreno, F. Cholestasis following chronic alcohol consumption: Enhancement after an acute dose of chlorpromazine. *Biochem Biophys Res Commun*, 94:1013-1020, 1980. Copyright 1980 by Academic Press Inc. [NY].)

Figure 6. Possible Role of Alcohol Modifying the Detoxifying and Toxifying Pathways of a Chemical Compound to its Product(s)

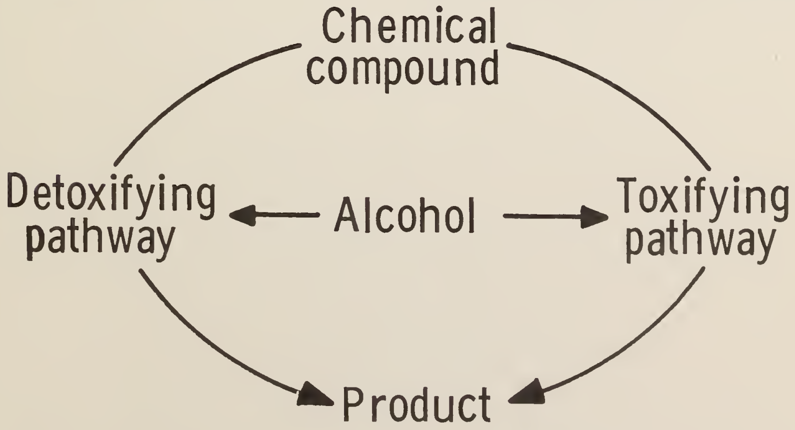
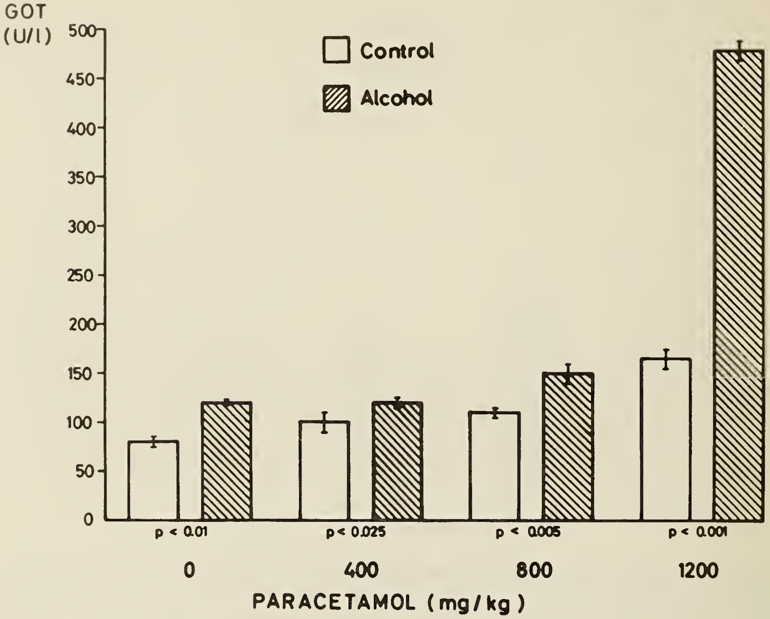
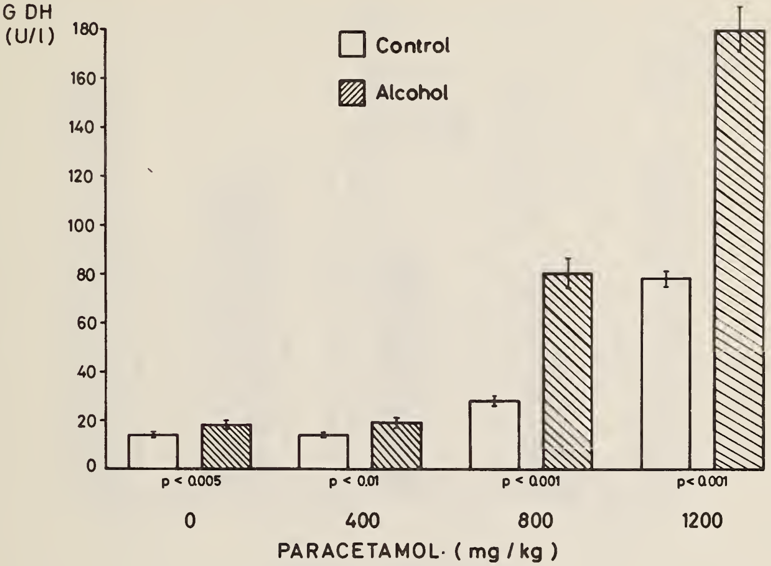


Figure 7. Effect of an Acute Dose of Paracetamol on Serum GOT Activity Following Chronic Alcohol Consumption



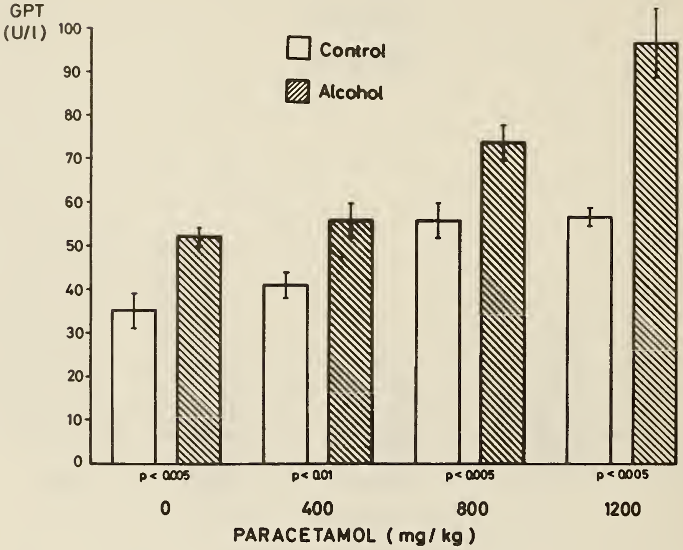
Rats were pair-fed for 4 weeks diets containing ethanol, whereas the control diet contained additional carbohydrates instead of ethanol. Eighteen hours after ethanol withdrawal, paracetamol was administered intraperitoneally at a dose indicated, and serum GOT activity was determined 18 hours thereafter. Each experimental group consisted of six animals (Teschke et al. 1979).

Figure 8. Effect of an Acute Dose of Paracetamol on Serum GDH Activity Following Chronic Alcohol Consumption



The experimental conditions are given in the legend for figure 7. (From Teschke, R.; Stutz, G.; and Strohmeyer, G. Increased paracetamol-induced hepatotoxicity after chronic alcohol consumption. *Biochem Biophys Res Commun*, 91:368-374, 1979. Copyright 1979 by Academic Press Inc. [NY].)

Figure 9. Effect of an Acute Dose of Paracetamol on Serum GPT Activity Following Chronic Alcohol Consumption



The experimental conditions are given in the legend for figure 7. (From Teschke, R.; Stutz, G.; and Strohmeyer, G. Increased paracetamol-induced hepatotoxicity after chronic alcohol consumption. *Biochem Biophys Res Commun*, 91:368-374, 1979. Copyright 1979 by Academic Press Inc. [NY].)

Figure 10. Possible Mechanism of Increased Paracetamol-Induced Hepatotoxicity Following Chronic Alcohol Consumption

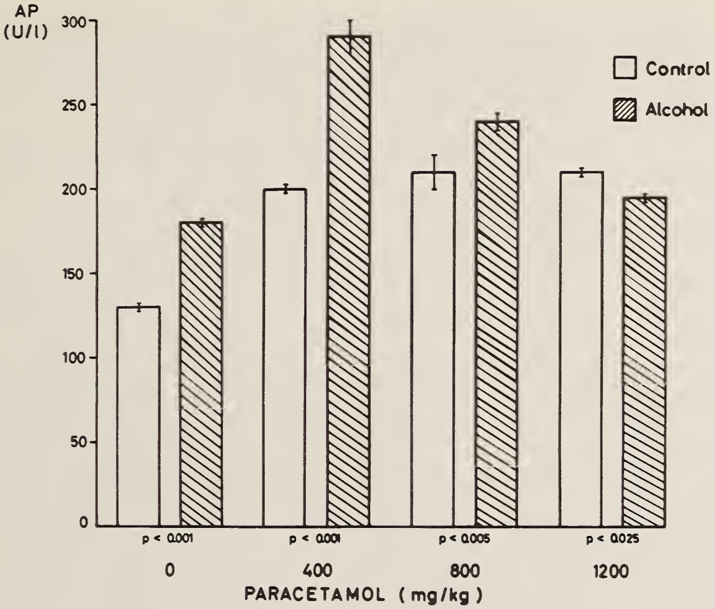


Figure 11. Effect of an Acute Dose of Paracetamol on Serum AP Activity Following Chronic Alcohol Consumption

Chronic alcohol consumption

↓

Proliferation of the smooth endoplasmic reticulum of the hepatocyte

↓

Microsomal enzyme induction

↓

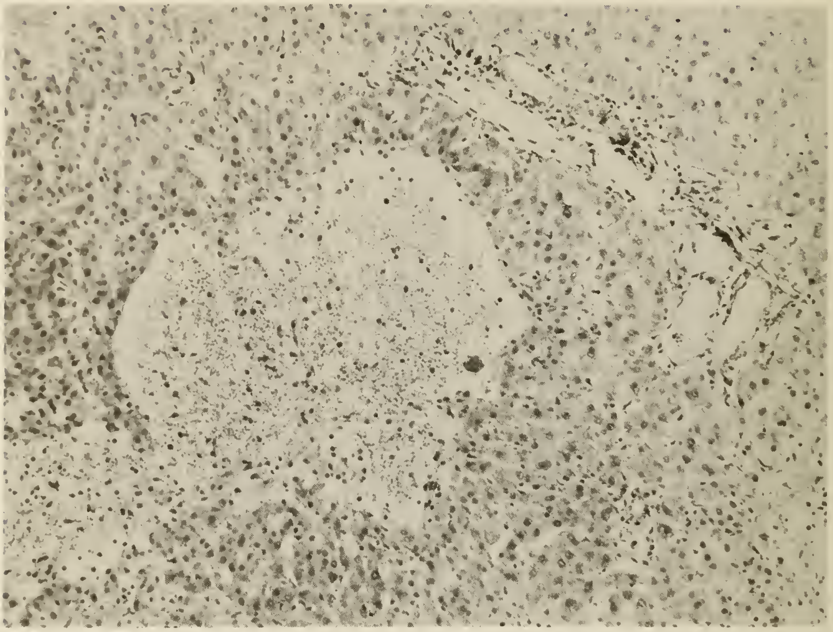
Increased microsomal metabolism of paracetamol to toxic intermediates

↓

Enhancement of hepatotoxicity

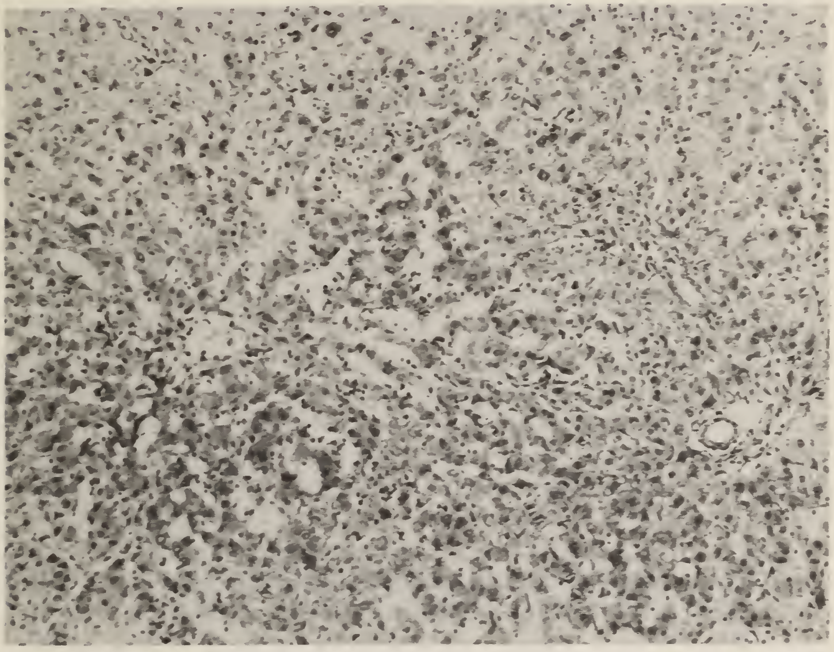
The experimental conditions are given in the legend for figure 7. (From Teschke, R.; Stutz, G.; and Strohmeyer, G. Increased paracetamol-induced hepatotoxicity after chronic alcohol consumption. *Biochem Biophys Res Commun*, 91:368-374, 1979. Copyright 1979 by Academic Press Inc. [NY].)

Figure 12. Liver Histology of Treatment with DMN Following Control Diet



DMN (1 mg/100 g BW) was administered intraperitoneally on 5 consecutive days after pretreatment of the rats with the control liquid diet for 23 days. (From Gellert, J.; Moreno, F.; Haydn, M.; Oldiges, H.; Frenzel, H.; Teschke, R.; and Strohmeyer, G. Decreased hepatotoxicity of dimethylnitrosamine [DMN] following chronic alcohol consumption. In: Thurman, R.G., ed. *Alcohol and Aldehyde Metabolizing Systems. IV. Advances in Experimental Medicine and Biology*, 132:237-243, 1980. Copyright 1980 by Plenum Press.)

Figure 13. Liver Histology of Treatment with DMN Following Alcohol Diet



DMN (1 mg/100 g BW) was administered intraperitoneally on 5 consecutive days after pretreatment of the rats with the alcohol liquid diet for 23 days. (From Gellert, J.; Moreno, F.; Haydn, M.; Oldiges, H.; Frenzel, H.; Teschke, R.; and Strohmeyer, G. Decreased hepatotoxicity of dimethylnitrosamine [DMN] following chronic alcohol consumption. In: Thurman, R.G., ed. *Alcohol and Aldehyde Metabolizing Systems. IV. Advances in Experimental Medicine and Biology*, 132:237-243, 1980. Copyright 1980 by Plenum Press.)

Table 1. Effect of Chronic Ethanol Consumption on the Activities of Glutamate Dehydrogenase (GDH), Glutamate-Oxalacetate-Transaminase (GOT), and Glutamate-Pyruvate-Transaminase (GPT) in the Serum After Repeated Administration of Dimethylnitrosamine (DMN)

Enzyme Activity	DMN	Control Rat	Ethanol Rat	p
GDH	+	29.5 ± 3.6	11.5 ± 1.3	<0.0025
(units/l)	−	6.4 ± 1.8	9.8 ± 1.3	N.S.
GOT	+	212.0 ± 25.0	164.0 ± 14.0	<0.05
(units/l)	−	145.0 ± 15.0	177.0 ± 16.0	<0.05
GPT	+	180.0 ± 14.0	124.0 ± 7.0	<0.005
(units/l)	−	81.0 ± 7.0	80.0 ± 7.0	N.S.

The Effects of Acute and Chronic Ethanol Administration on the Hepatotoxicity of Acetaminophen

Chifumi Sato, Yoshiro Matsuda, Masayuki Nakano, and Charles S. Lieber

Acetaminophen, a widely used analgesic and antipyretic, has been shown to produce hepatic injury after its biotransformation to reactive metabolite(s) by mixed function oxidation (Jollow et al. 1973; Mitchell et al. 1973; Potter et al. 1973). The interaction of ethanol intake and acetaminophen-induced hepatotoxicity has been of clinical interest because recent clinical reports suggest the susceptibility of alcoholics to the hepatotoxicity of acetaminophen (Emby and Fraser 1977; Wright and Prescott 1973). Besides its major metabolizing pathways through glucuronidation and sulfatation, acetaminophen has been shown to be metabolized by mixed function oxidation resulting in the production of reactive metabolite(s). The reactive metabolite(s) produced are detoxified by binding with reduced glutathione (GSH), followed by the excretion into the urine as mercapturic acid. When this process becomes insufficient, the reactive metabolite(s) can no longer be detoxified by this pathway, and they bind to cellular macromolecules. This covalent binding has been postulated to produce hepatic injury (Mitchell and Jollow 1975).

Interaction of ethanol with microsomal drug metabolizing system is complex. Acute ethanol administration inhibits drug oxidation in vivo and in vitro (Rubin and Lieber 1968; Rubin et al. 1970), whereas chronic ethanol consumption increases a variety of drug metabolizing enzyme activities (Misra et al. 1971; Rubin and Lieber 1968). Therefore, theoretically, interaction of ethanol with acetaminophen-induced hepatotoxicity may not be simple. To study the respective roles of acute and chronic ethanol administration, we studied acetaminophen-induced hepatotoxicity after withdrawal from chronic ethanol consumption (in the absence of ethanol) and after acute ethanol administration (in the presence of ethanol).

To study the chronic effect of ethanol, male Sprague-Dawley rats (130 to 150 g body weight [BW]) were pair-fed a nutritionally adequate liquid diet containing either ethanol as 36 percent of total calories or isocaloric carbohydrate for 4 to 6 weeks. After a 12-hour

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NOTE: Figures appear at end of paper.

fast, rats were injected with acetaminophen (0.5 g/kg intraperitoneally). By 36 hours, frank hepatic necrosis and a decrease in hepatic aminopyrine N-demethylase activity were observed in ethanol-fed rats, whereas in controls the changes were minimal. Serum GOT and glutamate dehydrogenase activities were significantly increased in ethanol-fed rats (figure 1). Although there was no statistical difference in hepatic GSH content before acetaminophen, the depletion of GSH after acetaminophen was significantly greater in ethanol-fed rats than in controls. Covalent binding of reactive metabolite(s) of acetaminophen to hepatic proteins in vivo was greater in ethanol-fed rats than in controls. Urinary excretion of mercapturic acid during the first 12 hours was also increased in ethanol-fed rats. In an in vitro study, covalent binding of reactive metabolite(s) of acetaminophen to microsomal protein (NADPH-dependent) was increased after ethanol feeding for 4 to 6 weeks. These findings suggest that (a) chronic ethanol consumption increases acetaminophen-induced hepatotoxicity, and (b) enhanced microsomal production of reactive metabolite(s) may be responsible for this effect.

To study the effect of acute ethanol, we used young male S-D rats (90 to 130 g BW) because we found that young rats are more susceptible to acetaminophen-induced hepatotoxicity than are older rats due to their lower hepatic GSH content. The rats were fasted for 18 hours and were given ethanol (6 g/kg) or saline by gastric tube. Six hours thereafter, the animals were injected with acetaminophen (0.5 g/kg) intraperitoneally. Acetaminophen produced hepatic injury in saline controls as judged by serum enzyme activities (GOT, GPT, and GDH; see figure 2) and histology, but in ethanol-treated rats, these changes were minimal. Decreased hepatotoxicity after ethanol was preceded by lesser covalent binding of reactive metabolite(s) of acetaminophen to hepatic proteins, although there was more unmetabolized acetaminophen in the liver. Six hours after ethanol or saline, there was no difference in hepatic GSH content in this experimental model. At this point, mixed function oxidase activity (measured in vitro using acetaminophen as substrate) was similar between these two groups. After acetaminophen, GSH depletion was more striking in saline controls than in ethanol-treated rats. In isolated hepatocytes, the depletion of GSH caused by acetaminophen was partially prevented by the presence of ethanol. Covalent binding of reactive metabolite(s) of acetaminophen to microsomal protein (NADPH-dependent) as well as acetaminophen-induced spectral change in microsomes was inhibited in the presence of ethanol. These data suggest that (a) acute ethanol administration prevents acetaminophen-induced hepatotoxicity, and (b) this effect may be due to the inhibition of biotransformation of acetaminophen to reactive metabolite(s).

These various experiments support clinical observations that suggest that alcoholics are particularly susceptible to acetaminophen-induced hepatotoxicity; however, when ethanol is taken together with acetaminophen, hepatotoxicity might be prevented (Rumach et al. 1981).

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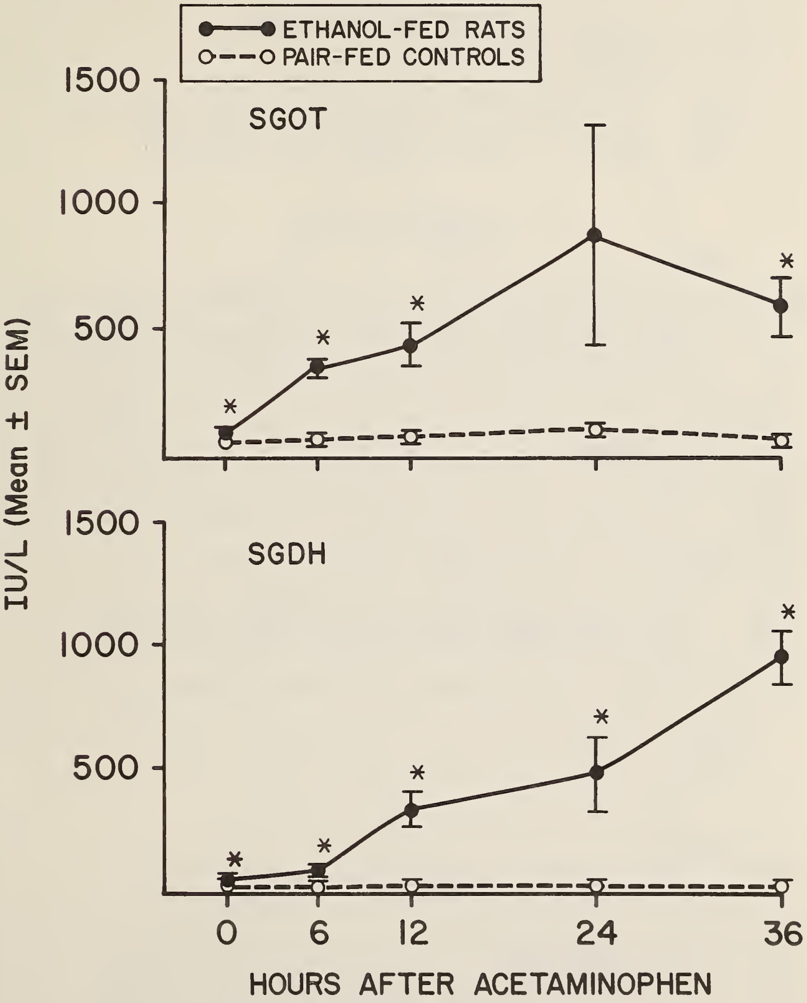
Sato, C.; Matsuda, Y.; and Lieber, C.S. Increased hepatotoxicity of acetaminophen after chronic ethanol consumption in the rat. *Gastroenterology*, 80:140-148, 1981.

Sato, C.; Nakano, M.; and Lieber, C.S. Prevention of acetaminophen-induced hepatotoxicity by acute ethanol administration in the rat: Comparison with carbon tetrachloride-induced hepatotoxicity. *J Pharmacol Exp Ther*, in press.

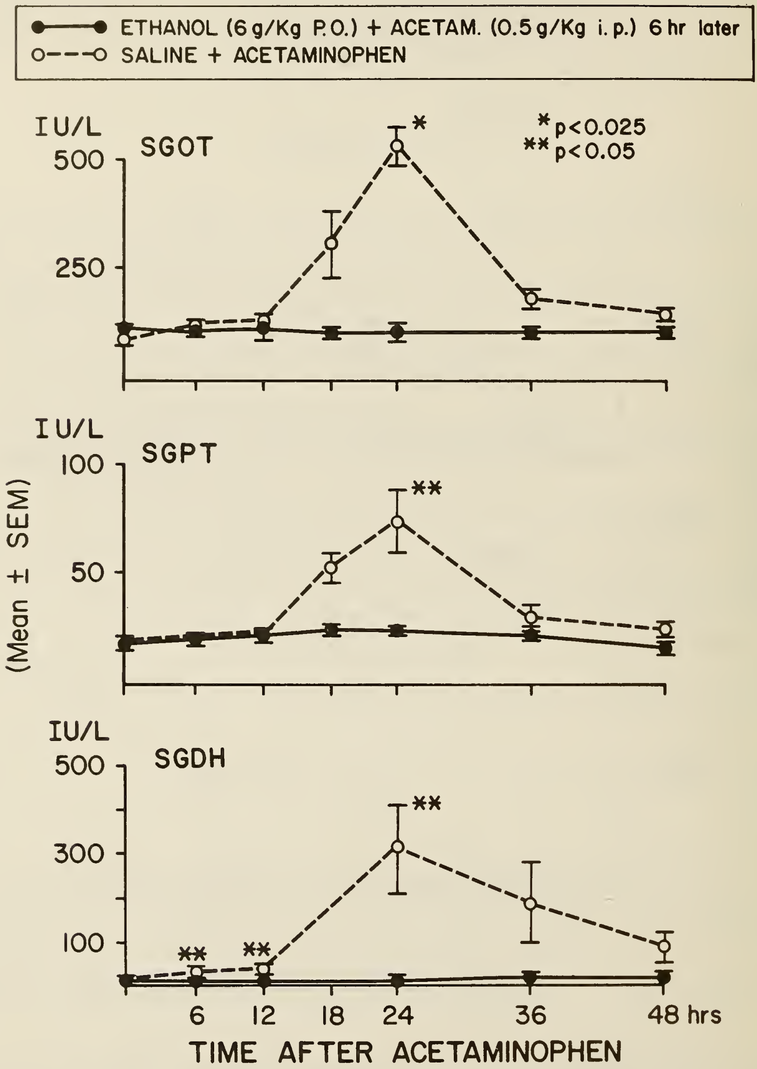
Wright, N., and Prescott, L.F. Potentiation of previous drug therapy of hepatotoxicity following paracetamol overdosage. *Scot Med J*, 18:56-58, 1973.

Figure 1. Time Course of Serum Enzyme Activities After the Injection of Acetaminophen



Male rats (130 to 150 g BW) were pair-fed a liquid diet containing ethanol or isocaloric carbohydrate for 4 to 6 weeks (280 to 360 g BW). After a 12-hour fast, the rats were given acetaminophen (0.5 g/kg, intraperitoneally). Each point represents the mean value of at least five animals (*$p<0.05$). (From Sato, C.; Matsuda, Y.; and Lieber, C.S. Increased hepatotoxicity of acetaminophen after chronic ethanol consumption in the rat. *Gastroenterology*, 80:140-148, 1981. Copyright 1981 by Elsevier North-Holland, Inc. [NY].)

Figure 2. Effect of Acute Ethanol Administration on the Rise of Serum Enzyme Activities



Young male rats (90 to 130 g BW) were fasted for 18 hours and were given ethanol (6g/kg) or saline by gastric tube. Six hours after the treatment, they were injected with acetaminophen (0.5 g/kg, intraperitoneally). Each point represents 6 to 10 determinations. (From Sato, C.; Nakano, M.; and Lieber, C.S. Prevention of acetaminophen-induced hepatotoxicity by acute ethanol administration in the rat: Comparison with carbon tetrachloride-induced hepatotoxicity. *J Pharmacol Exp Ther,* in press. Copyright 1981 by the Williams and Wilkins Co. [Baltimore].)

Exacerbation by Ethanol of Liver Injury Due to Vitamin A Deficiency and Excess*

Maria A. Leo, Masao Arai, Mayumi Sato, and Charles S. Lieber

Introduction

Some patients with alcoholic liver disease have evidence of vitamin A deficiency as indicated by a low concentration of vitamin A in the blood (McClain et al. 1979; Smith et al. 1975). Furthermore, it has been suggested that lack of vitamin A may be involved in the pathogenesis of Mallory bodies, since the latter contain prekeratin (Denk et al. 1979; French 1981). The hepatic level of vitamin A in patients with alcoholic liver disease, however, including those with Mallory bodies, is not known. Therefore, the present study was undertaken to determine the level of hepatic vitamin A in patients with alcoholic and other types of liver diseases and to assess whether vitamin A deficiency contributes to the development of liver lesions. The role played by vitamin A in the pathogenesis of liver pathology, independently of other factors, was then verified in the rat under strictly controlled dietary conditions. The same experimental model also was used to study the potential hepatotoxicity of vitamin A supplementation.

Indeed, when taken in large amounts, vitamin A has been shown to be toxic both in humans and in experimental animals (Farrell et al. 1977; Lane 1968; Russell et al. 1974). In moderate doses, however, vitamin A is used to correct various clinical conditions such as abnormal dark adaptation (Russell et al. 1978) or hypogonadism (McClain et al. 1979). These commonly used amounts of vitamin A are considered safe because no adverse effects have been reported when they were administered to normal individuals. Their safety in alcoholics, however, has not been established, and the question must be raised whether the alcoholic may develop some unusual susceptibility to the hepatotoxicity of vitamin A. Indeed, it has been shown that in the case of other hepatotoxic agents, such as carbon tetrachloride (Hasumura et al. 1974) and acetaminophen (Sato et al. 1981). Chronic consumption of ethanol strikingly exacerbates the hepatotoxicity of these

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NOTE: Figures and tables appear at end of paper.

* Studies were supported by U.S. Public Health Service grant AA-03508 and the Veterans Administration.

agents. Therefore, it is conceivable that potential hepatotoxic agents such as vitamin A, when used in amounts that are innocuous in normal individuals, may exert hepatotoxic effects in the alcoholic. To study this question, we investigated the hepatic effects of moderate vitamin A supplementation in control and alcohol-fed rats.

Materials and Methods

Human Studies

Liver tissues were obtained from 52 needle biopsies performed for diagnostic purposes and from 13 autopsies carried out less than 12 hours postmortem. The patients varied in age from 20 to 60 years and were all males. Forty-one had alcoholic and 17 had nonalcoholic diseases (figure 1). The latter group comprised 7 drug-induced hepatitis and 10 other etiologies: 2 granulomatous hepatitis, 2 viral and 2 persistent hepatitis, 1 lymphoma, 2 with chronic passive congestion, and 1 fatty liver secondary to postgastrectomy malabsorption.

A liver aliquot was immediately frozen in dry ice for vitamin A determination, and another portion of liver was fixed in 10 percent buffered formalin for routine light microscopy. In 42 patients, tissue was also available for electron microscopy. Blood was drawn in 33 fasted patients at the time of the liver biopsy for serum vitamin A, retinol-binding protein (RBP), and prealbumin (PA) determinations.

Animal Studies

Male Sprague-Dawley rats were fed a liquid diet containing adequate amounts of proteins, minerals, and vitamins (Lieber and DeCarli 1970) with a normal content of vitamin A (5,800 IU/l as retinyl acetate, with an average daily total vitamin A intake of 400 IU); or with a low vitamin A diet, with a 300-fold decrease; or with a high vitamin A diet, with a fivefold increase in vitamin A content. After weaning, the littermates were pair-fed either one of the control diets or the corresponding diet in which carbohydrates (36 percent of total calories) were isocalorically replaced by ethanol. After 60 days, all animals were sacrificed under ether anesthesia after an overnight fast. Blood was collected from the abdominal aorta. The liver was removed and quickly frozen in dry ice for vitamin A determination. Samples were taken for light microscopy and electron microscopy.

Liver and serum were stored at −80°C in the dark until analyzed for vitamin A content. Liver tissue was also obtained from 21 normal baboons housed in our colony.

Vitamin A Assay

Human and baboon liver samples (approximately 10 mg for biopsy specimens and 200 to 300 mg for autopsy samples) were extracted with chloroform-methanol (Folch et al. 1957). Rat liver tissue (0.5 to 1 g) was extracted with diethyl ether by the method of Ames et al. (1954). Recovery with the Folch extraction was 95 percent, and no difference was found between the two methods. Vitamin A was measured by the trifluoroacetic acid method of Dugan et al. (1964). Retinyl palmitate was used as a standard. The amount of vitamin A in the samples was expressed as the equivalent weight of all-trans retinol. The serum samples were assayed for total vitamin A by the fluorometric method of Thompson et al. (1971) with the addition of butylated hydroxytoluene to the n-hexane at a concentration of 20 μg/ml as an antioxidant. Retinol was used as a standard.

Retinol-Binding Protein (RBP) and Prealbumin (PA) Determinations

Human serum retinol-binding protein (RBP) and prealbumin (PA) were assayed by radial immunodiffusion using the Calbiochem-Behring M-Partigen Kits. Human serum was used as a standard.

Rat RBP was isolated from serum (type I), and antiserum was prepared from rabbits according to Muto and Goodman (1972) as previously described (Sato and Lieber 1981). The rat serum RBP was determined by the radial immunodiffusion method of Mancini et al. (1965).

VLDL Studies

Lipoproteins were fractionated according to Bronzert and Brewer (1977), and VLDL triglycerides were measured according to Pinter et al. (1967). Aliquots of the VLDL fractions were negatively stained with a 2 percent solution of phosphotungstic acid, adjusted to pH 7.1 with potassium hydroxide (Strange et al. 1975) and examined under an electron microscope.

Mitochondrial Preparation

Liver mitochondria were prepared according to Beattie (1968) with the modification of Paterniti and Beattie (1979), utilizing 0.25 M sucrose buffered with 10 mM tris-Hcl (pH 7.4) and 1 mM EDTA as isolation medium. The mitochondrial pellet was washed twice with the media containing 0.5 percent albumin (fatty acid free) and then with the isolation medium. The pellet was finally suspended in 0.225 M sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 mM KCl, and 20 mM triethanolamine buffer (pH 7.4) (1 ml/g of liver). Mitochondrial recovery was 40 to 50 percent as calculated from the GDH content of the liver homogenate and mitochondrial pellet. The purity of the fraction was controlled by electron microscopy. A portion of the mitochondrial suspension was used for respiration studies; the remainder was frozen at −80°C and used for enzyme assays and for the determination of cytochrome contents. Protein concentration was determined by the method of Lowry et al. (1951), using bovine albumin as a standard.

Mitochondrial Respiration

Oxygen consumption was measured polarographically at 30°C with a Clark oxygen electrode. The mitochondrial suspension (3 to 5 mg mitochondrial protein) was added to 3 ml of respiration medium containing 0.225 M sucrose, 10 mM potassium phosphate, 5 mM $MgCl_2$, 20 mM KCl, and 20 mM triethanolamine buffer (pH 7.4), as outlined by Estabrook (1967). The capacity to oxidize substrates entering sites 1, 2, and 3 was measured. The final concentrations of substrates were 3.3 mM glutamate, 3.3 mM succinate, and 5 mM ascorbate plus 0.2 mM NNN′N′-tetramethyl-p-phenylenediamine. When 15 μM palmitate and 100 μM ATP plus 3 mM carnitine or 7 μM patmitoyl-CoA plus 3 mM carnitine were used as substrates, 0.2 mM malate and 6 mg bovine serum albumin (fatty acid free) were added to the respiration medium (Cederbaum et al. 1975). State 3 respiration was induced by addition of 150 μM ADP. ADP:O ratio and respiratory control ratio (RCR) were calculated according to Estabrook (1967).

Respiratory Chain Enzyme Assay

Freshly thawed mitochondria were used in all assays. The assay medium was preincubated for 2 min, and the reaction was initiated by addition of the enzyme. In each assay, the final concentration of mitochondrial protein was 20 to 40 μg/ml. The following enzyme activities were measured spectrophotometrically using a Cary 219 spectrophotometer:

- NADH dehydrogenase activity (NADH:$K_3Fe(CN)_6$ reductase) at 30°C following the reduction of ferricyanide at 420 nm. The assay mixture contained 1 mM NADH, 0.5 mM $K_3Fe(CN)_6$, 6 μM antimycin

A_3, and 50 mM potassium phosphate buffer (pH 7.4) (Minakami et al. 1962).

- Succinate dehydrogenase activity (succinate: DICP reductase) according to King (1967). The assay mixture contained 40 mM succinate, 0.9 mM phenazine methosulfate, 50 μM 2,6-dichlorophenolindophenol, 1.5 mM potassium cyanide, 0.1 percent bovine serum albumin, and 50 mM potassium phosphate buffer (pH 7.4).
- Cytochrome c reductase activity (with various substrates) at 37°C by following the reduction of cytochrome c at 550 nm (Hatefi and Rieske 1967; Sottocasa et al. 1967). The assay mixture contained 50 μM ferricytochrome c, 1.5 mM potassium cyanide, and 50 mM potassium phosphate buffer (pH 7.4). The substrates used were 1 mM NADH, 3.3 mM β-hydroxybutyrate plus 1 mM NAD or 3.3 mM succinate.
- Cytochrome oxidase activity at 37°C by following the oxidation of the reduced cytochrome c at 550 nm as described by Wharton and Tzagoloff (1967). The assay mixture contained 0.081 mM ferrocytochrome c and 50 mM potassium phosphate buffer (pH 7.4). As reference for the assay, ferrocytochrome c oxidized with ferricyanide was used.

Cytochrome Content of the Mitochondria

The cytochrome content was determined according to Williams (1964). The freshly thawed mitochondrial suspension was mixed with deoxycholate (1 mg/mg of protein). The solubilized mitochondria were divided in two aliquots of 1 ml each. In one aliquot, the cytochromes were reduced with 0.1 ml of 0.05 M ascorbate and a few grains of sodium dithionite, and the other was oxidized with 0.1 ml of 0.05 M sodium ferricyanide. The difference spectra were recorded from 500 to 650 nm wave length using an Aminco DW-2 spectrophotometer. Millimolar extinction coefficients of 21.0 $\text{mmoles}^{-1}\ \text{cm}^{-1}$ for cytochrome c (535-550nm), 15.6 $\text{mmoles}^{-1}\ \text{cm}^{-1}$ for cytochrome c_1 (540-554nm), 13.8 $\text{mmoles}^{-1}\ \text{cm}^{-1}$ for cytochrome b (563-577nm), and 12.0 $\text{mmoles}^{-1}\ \text{cm}^{-1}$ for cytochrome aa_3 (605-630nm) were used for the calculations of cytochrome contents.

Miscellaneous Determinations

Liver glutamate dehydrogenase (GDH) activity was measured according to Tottmar et al. (1973). The reaction mixture contained 50 mM KH_2PO_4 (pH 7.4), 120 mM ammonium acetate, 0.15 mM NADH, and 1.5 mM ADP. After addition of 0.1 percent (v/v) Triton

X-100 and a 5 min incubation at 25°C, the reaction was started by the addition of 8 mM alphaketoglutarate, and readings from the Cary Spectrophotometer were taken at 340 nm.

Serum GDH activity was assayed by the method of Ellis and Goldberg (1972) at 37°C. Total hepatic lipids were extracted according to Folch et al. (1957) and measured by the method of Amenta (1964). Liver triglycerides were measured by the method of Snyder and Stephens (1959).

Electron Microscopy Studies

Liver tissue was cut into small pieces, fixed in 2.5 percent glutaraldehyde in cacodylate buffer for 3 hours and postfixed in 1 percent osmium tetroxide for 2 more hours. After dehydration the specimens were embedded in Epon 815 (Luft 1961). One-micron thick sections were stained with toluidine blue, and pericentral areas as well as periportal areas were selected for thin sections. Sections were cut with an LKB IV ultramicrotome and stained with uranyl acetate and lead citrate. Selected areas were examined under an Hitachi 8 electron microscope. In 10 rats fed the low vitamin A diet (5 alcohol-fed and 5 controls), the number of multivesicular lysosomes (MLL) were counted on randomly taken pictures of periportal areas.

Statistics

All results were expressed as mean ± standard error of the mean. Statistical significance was analyzed by Student's group t test, unless otherwise stated (Snedecor and Cochran 1967).

Results

Human Studies

Vitamin A levels measured in 65 human liver specimens are shown in figure 1. Low levels were observed in various disease states, particularly alcohol- and drug-induced liver injury. The values were much lower than those measured in 2 normal livers (figure 1). Only these 2 subjects with normal livers were available for vitamin A determination; although widely different, their values fell within the range of the results observed in 21 baboons with normal livers in which vitamin A varied from 478 to 2376 μg/g wet weight, with a mean of 1272±102.4 μg/g.

Mallory bodies were observed in 9 subjects with alcoholic hepatitis and/or cirrhosis. Their vitamin A values ranged widely from subnormal to negligible levels. The range and the mean were not different from the values observed in 24 patients (17 subjects with alcoholic hepatitis and/or cirrhosis and 7 with drug-induced liver disease) without Mallory bodies (figure 1).

On the average, patients with simple fatty liver had values higher than those with more severe liver diseases.

Blood was available for vitamin A, retinol-binding protein (RBP), and prealbumin (PA) measurements in 35 subjects (table 1). In patients with nonalcoholic liver diseases and alcoholic fatty livers, vitamin A levels in the liver were depressed, but this occurrence was not associated with lower blood levels (table 1). In the patients with alcoholic hepatitis and/or cirrhosis, there was a significant decrease in serum vitamin A, RBP, and PA, whereas in the other subjects the values were close to values previously reported as normal by Smith and Goodman, namely 50.0±1.5 μg/100 ml for vitamin A, 46.2±1.0 μg/ml for RBP, and 250±5 μg/ml for PA (Smith and Goodman 1971).

By electron microscopy, multivesicular lipid containing lysosome-like structures (MLL) (figure 2) were seen in all patient groups in which lowered hepatic vitamin A was observed (figure 3). These organelles were common in periportal areas and near the bile ductules. They were limited by a single membrane with a homogeneous electron dense crescent and contained numerous lipid-like particles of different sizes, some of which were reminiscent of lipoprotein particles (figure 2). These structures were seen primarily in hepatocytes, but they were also found in macrophages (figure 4).

No normal human liver tissue was available for electron microscopy, but in normal rats and baboons MLLs were never identified. Since all patients with the lesion had in common a moderately or severely depressed hepatic vitamin A level, the hypothesis that low vitamin A may favor the development of multivesicular lysosomes was tested in rats fed a low vitamin A diet.

Animal Studies

Effect of Vitamin A Depletion

Compared to animals fed the normal vitamin A containing diet, rats given the control vitamin A deficient diet had only trace amounts of vitamin A in the liver and decreased vitamin A and RBP in the blood (table 2).

Ethanol feeding resulted in a decreased level of hepatic vitamin A in the group fed the normal diet and a depletion to an unmeasurable level in rats fed the low vitamin A diet. By light microscopy, no lesions were observed in control rats fed the low vitamin A diet, but by electron microscopy, MLL were found in all animals. They were abundant in periportal zones and congregated in pericanalicular areas. The lesions were identical to those seen in the patients; they contained round lipid-like particules the smallest of which resembled in size and shape the particles found in the secretory vesicles of the Golgi and the plasma VLDL of these animals examined by electron microscopy after negative staining. When ethanol was added to the low vitamin A diet, the lesions became more numerous and more enlarged (figure 5), compared to the control (figure 6). Some of these structures had mitochondria that were two to three times the size of an average mitochondrion. The number of MLL was $0.058 \pm 0.008/\mu m^2$ in five alcohol-fed rats and $0.022 \pm 0.008/\mu m^2$ in the corresponding controls ($p < 0.02$). The lesions were seen also in pericentral areas of animals fed the control (figure 6) and alcohol-containing low vitamin A diet. As in the case of human tissue, MLL were most abundant in hepatocytes, but they were also seen in macrophages.

Besides the lysosomal alterations, the changes previously ascribed to ethanol, such as giant mitochondria and proliferation of the endoplasmic reticulum, were also seen in these animals, particularly in the pericentral areas. Rats or baboons fed a normal vitamin A diet with or without ethanol did not display any of these lysosomal changes.

To test the effect of vitamin A depletion on circulating VLDL, serum lipoproteins were fractionated in all groups. Fasting VLDL triglycerides were lower in vitamin A depleted rats. Furthermore, whereas ethanol when given with a normal vitamin A diet was associated with increased fasting VLDL triglycerides, the opposite was the case in the animals fed the low vitamin A diet.

Effects of Vitamin A Supplementation

Vitamin A levels in liver and serum are shown in table 3. Compared to the normal A diet, after high A, there was a striking increase of the vitamin A level in the liver. It is noteworthy that in the alcohol-fed animals the rise in vitamin A in the liver was much less striking than in control animals fed the high A diet. Even when the total hepatic vitamin A was expressed per 100 g body weight (BW), the content was lower after ethanol despite the ethanol-induced hepatomegaly, both in animals fed the normal A and the high A diets. It also is noteworthy that although the high A control had a twofold increase in hepatic vitamin A (compared to the normal A control), after ethanol even the high A had a lower liver vitamin A concentration than did the normal A control. Retinol-binding protein in the serum of the high vitamin A control rats was 39.1±4.0 μg/ml, a value not significantly different from that obtained in rats fed the normal vitamin A diet (30.7±4.4 μg/ml); it was unaffected by the feeding of the combination of high vitamin A and ethanol (41.0±2.3 μg/ml). After normal vitamin A and ethanol, the value was 45.7±6.3 μg/ml.

Mitochondrial Respiration

The effects of ethanol and/or vitamin A on mitochondrial respiration are shown in figure 7. With all substrates, oxygen consumption in control animals was unchanged by the feeding of a high vitamin A diet, whereas after ethanol oxygen consumption was depressed with both diets, but significantly more with the high A than with the normal A diet (figure 7). Respiratory control (figure 8) and ADP:O ratios (figure 9) showed similar changes with all substrates except palmitate. With palmitate, the values were lower after high A control than normal A control. Comparable but less striking changes were found in state 3 respiration.

Chemical Changes in Mitochondria

As shown in table 4 and figure 10, alcohol feeding resulted in a depression of mitochondrial enzyme activities, measured both in rats fed the normal A diet and those given the high A diet. There was a tendency for the effect to be more striking after high A and ethanol, but the difference was significant only for succinate-cytochrome c reductase and cytochrome c oxidase activities (figure 10). Except for cytochrome c oxidase activity (figure 10), there was no significant difference between the normal A and high A control results. Ethanol feeding significantly depressed mitochondrial glutamate dehydrogenase activity (per mg protein) in rats fed both the normal and the high vitamin A diets (table 5). Even calculated per total liver, GDH again was reduced by ethanol in the high A group, with a comparable (but not significant) difference in the rats fed the normal A diet.

As shown in table 6, vitamin A by itself significantly depressed only cytochrome aa_3 content. Ethanol by itself depressed both cytochrome aa_3 and cytochrome b. The reduction of the latter was most pronounced when ethanol was given with the high vitamin A diet; under these conditions, cytochrome $c + c_1$ was also depressed.

Various Chemical Changes

Serum GDH tended to be higher after ethanol treatment in both the normal A group (8.1±3.3 versus 3.7±0.5 IU/L) and the high A group (33.0±14.0 versus 4.1±0.3 IU/L). Because of the considerable variations between pairs of littermates, the results were not significantly different when calculated according to Student's group t test. The difference became significant, however, when the p value was calculated according to Student's paired t test of ethanol/control ($p<0.01$).

After alcohol administration, liver total lipids were increased both in the normal A (76.9±8.1 versus 45.9±3.4 mg/g; $p<0.01$) and in the high A group (112.4±18.8 versus 52.3±3.0; $p<0.01$). The corresponding triglycerides values were 31.4±7.5 versus 9.6±1.9 mg/g ($p<0.02$) after normal A and 64.1±18.3 versus 11.9±3.4 ($p<0.01$) after high A.

Morphologic Changes

By light microscopy, the livers of the animals fed the normal A control diet were normal; rats fed the normal A and ethanol diet developed moderate steatosis, as described before (Lieber et al. 1965). After the high A control diet, no significant changes were seen, whereas in the high A and ethanol diet, steatosis appeared to be somewhat more striking than in the normal A and ethanol group. In paraffin sections stained with haematoxilin-eosin, mitochondria are not identifiable with certainty; however, in toluidine-blue stained Epon thick sections they are easily discerned. In rats fed the normal A and ethanol diet, several spheroidal and enlarged mitochondria were identified in the hepatocytes (figure 11a). By contrast, in the high A and ethanol group, usually giant mitochondria, often exceeding in size the diameter of the nucleus, were found in the hepatocytes (figure 11b). The presence of numerous enlarged and giant mitochondria in a great number of hepatocytes in the high A and ethanol group clearly differentiated that group from the normal A and ethanol group. More severe fat accumulation was also present in the high A and ethanol group, as illustrated in figure 11b. Such accumulation was confirmed on frozen sections stained with Oil Red O. The control rats fed either normal or high vitamin A diets showed virtually no difference by light microscopy. Fluorescent microscopy of unstained frozen sections of the high vitamin A groups (ethanol or pair-fed controls) showed rapidly fading green autofluorescence in the perisinusoidal space, presumably due to vitamin A (Popper 1944).

By electron microscopy, the normal A control group showed no liver abnormality (figure 12a), whereas the normal A ethanol group displayed the anticipated moderate mitochondrial alterations and the proliferation of the smooth endoplasmic reticulum, as described before (Iseri et al. 1966) (figure 12b). Moderate lesions were observed also in the high A control group consisting of swelling of the mitochondria, rarefaction of the matrix with relatively good preservation of the cristae and proliferation of the smooth endoplasmic reticulum (figure 13a). By contrast, all animals of the high A and ethanol group showed enlarged mitochondria with extremely variable sizes and striking giant figures, approaching and many times exceeding the size of the nucleus (figure 13b). Remnants of the cristae were still seen close to the mitochondrial membrane. The matrix was dense and often contained dark irregular paracrystalline inclusions. Proliferation of the smooth endoplasmic reticulum, enlarged lysosomes, and prominent "fat storing cells" were also seen in the high A group (ethanol as well as pair-fed controls).

Discussion

This study revealed that even in the presence of a normal blood level, vitamin A is commonly depressed in the liver of patients with various hepatic diseases. The levels were particularly low in alcohol- and drug-induced liver injury and were commonly associated with multivesicular lysosomal lesions. This study also shows that in the rat, treatment with ethanol greatly enhances the hepatotoxicity of vitamin A and that amounts of vitamin A which under normal conditions are virtually harmless may acquire unusual hepatotoxicity.

The mechanism whereby diseases of the liver are associated with low hepatic vitamin A levels is not clear. Malnutrition, when present, could of course contribute to hepatic vitamin A depletion. It must be pointed out, however, that in our patient materials the lowered amount of liver vitamin A was not necessarily associated with lowered blood levels (table 1). Furthermore, experimentally, administration of ethanol with normal or even enriched vitamin A diets resulted in a depression of hepatic vitamin A that could not be attributed to insufficient vitamin A intake of malabsorption (Sato and Lieber in press). Enhanced degradation of vitamin A in the liver after chronic alcohol consumption and/or possible enhanced mobilization of vitamin A from the liver have been postulated to explain at least in part the depression of vitamin A that was evident after ethanol consumption (Sato and Lieber in press).

Whatever the mechanism of vitamin A depletion in the liver, in view of the fact that very low levels were sometimes found, the question must be raised of the possible impact of this hepatic depletion of vitamin A on liver function and structure.

In subjects with alcoholic liver disease, we found very low levels of hepatic vitamin A to be sometimes associated with the presence of Mallory bodies. It must be noted, however, that Mallory bodies were seen also in patients with alcoholic liver disease who had relatively normal levels of vitamin A and that, conversely, patients with drug-induced liver disease and very low levels of vitamin A did not show any Mallory bodies. Therefore, the causal relationship between a lowered hepatic vitamin A and the appearance of Mallory bodies, postulated by Denk et al. (1979) and French (1981), was not verified, although the possibility has not been ruled out that a lowered vitamin A diet may potentiate some effect of ethanol that may result in the development of Mallory bodies. Although no obvious correlation was found between the appearance of Mallory bodies and the depletion of vitamin A in the liver, it was noted that in patients with severe as well as moderate depletion of hepatic vitamin A, multivesicular lysosome-like organelles were detected in increased numbers (figures 2 and 3). These structures had a single membrane with crescent previously described as characteristic of lysosomes (Hayashi et al. 1977). Such lysosomal lesions have not been related to low vitamin A levels before, but similar lesions have been described in patients with Wilson's disease (Hayashi et al. 1977; Tanikawa 1979) and in livers of rats treated with antimicrotubular drugs and perfused with oleic acid (Reaven and Reaven 1980). We did not have the opportunity to survey normal human livers with electron microscopy for the possible occurrence of such types of multivesicular lipid-containing lysosomes. To our knowledge, they have never been described in normal livers before, and we did not see this lesion in the livers of normal rats or baboons.

Many factors could contribute to the appearance of such lysosomes, including hepatic vitamin A depletion. The possibility that a lowered vitamin A level might contribute to these lesions was verified experimentally in the rat. It was discovered that whereas multivesicular lysosomes were not seen in the rats fed a control diet, administration of a low vitamin A diet resulted in the appearance of such lesions. Moreover, addition of ethanol to such a low vitamin A diet increased the number of the lesions. Thus, it appears reasonable to conclude that in patients with strikingly lowered hepatic vitamin A, the latter might contribute to the appearance of these multivesicular lysosomes.

The mechanism whereby a lowered vitamin A diet might produce these lesions is not known. These vesicles seem to be filled with numerous lipid-like particles that suggest that lipoprotein secretion might be involved. Indirect evidence in favor of such a hypothesis was provided by the observation of lowered circulating VLDL lipoproteins in rats fed the low vitamin A diet compared to animals fed the normal vitamin A regimen.

It is also noteworthy that vitamin A is required for glycosylation of export proteins (Wolf et al. 1979). Further studies are required to determine the link between lowered vitamin A and the appearance of the multivesicular lysosomes, including the possible involvement of a defect in lipoprotein metabolism. It is noteworthy that ethanol itself, even in the presence of an adequate vitamin A intake, can produce engorgement of the Golgi apparatus and the endoplasmic reticulum with lipoprotein-like particles (Matsuda et al. 1979). It is therefore not surprising that ethanol was found in the present study to exacerbate the lesions produced by vitamin A deficiency. It is conceivable that ethanol also favors the appearance of this lesion through its general effect of lipid retention in the liver and the associated steatosis (Lieber 1974). We must point out, however, that even in the absence of fatty liver in the animals fed a control low vitamin A diet, the lesions still appeared, but they were less severe than in the presence of ethanol.

Whether ethanol has direct effect on the lysosome itself has been the subject of controversy; both increases (Mezey et al. 1976) and decreases (Platt et al. 1971) of lysosomal enzymes after ethanol have been reported. Furthermore, alterations of lysosomal membranes have been described after the feeding of vitamin-A deficient diet in rats (Adhikari and Vakil 1980; Dingle 1966). Thus, it is not surprising that a combination of ethanol and low vitamin A resulted in the striking lysosomal abnormalities described in the present study.

In view of the common occurrence of low vitamin A levels in the livers of patients with liver disease described here and the possible relationship between lowered vitamin A and alteration of the liver functions and structure, the question of the therapeutic use of vitamin A in patients with liver disease, particularly alcoholics, must be raised. When manifest vitamin A deficiency is present (as corroborated by low vitamin A levels), such treatment might be particularly indicated. It is noteworthy, however, that even in the presence of normal serum vitamin A, the liver levels may be severely depressed (table 1). Whether vitamin A should be administered in these patients and, if so, how much should be given is still an open question. Indeed, although vitamin A deficiency can adversely affect the liver, an excess of vitamin A is also known to be hepatotoxic (Farrell et al. 1977; Russell et al. 1974). The amounts used were very large and exceeded greatly the dose of vitamin A commonly used for the treatment of several clinical conditions such as psoriasis (Fleischmann et al. 1977) and xerophthalmia (Sommer et al. 1980). Among other therapeutic usages, vitamin A is given to alcoholics with hypogonadism and abnormal dark adaptation (McClain et al. 1979). The amounts administered are considered safe because no adverse effects have been reported in normal individuals with these dosages. It must be pointed out, however, that in the case of other potential hepatotoxic agents such as acetaminophen, alcoholics were reported to have an unusual susceptibility to the drug in terms of hepatotoxicity (Lieber 1980), and in rats alcohol pretreatment has been shown to potentiate the hepatotoxicity of acetaminophen (Sato et al. 1981).

The present study shows that a similar potentiation also occurs with regard to vitamin A, which produced only mild effects in the livers of control animals, but when given to alcohol-fed animals induced ultrastructural and functional lesions of the mitochondria indicative of severe hepatotoxicity. In the amount used in the present study, vitamin A supplementation by itself produced merely moderate changes, characterized by some endoplasmic reticulum proliferation, slight enlargement of mitochondria, and moderate decrease in cytochrome c oxidase activity and cytochrome aa_3 content. Other mitochondrial enzymes were unaffected. The endoplasmic reticulum changes observed in this study after 8 weeks of moderate vitamin A supplementation are reminiscent of those described by Lane (1968) after 5 days of a massive dose of vitamin A. When ethanol was given with a normal vitamin A diet, the lesions observed in the present study were the same as those described before, namely, selective impairment of mitochondrial functions (Cederbaum et al. 1975; Rubin et al. 1970), some mitochondrial enlargement, and proliferation of the smooth endoplasmic reticulum (Iseri et al. 1966). However, when ethanol was given with the high vitamin A diet, the severity of the ethanol-induced lesions increased strikingly. Particularly impressive were the appearance of a large number of giant mitochondria and the disappearance of the cristae and appearance of paracrystalline inclusions. These lesions were much more severe than any of the changes described in the literature even after massive vitamin A treatment (in the absence of ethanol) (Hruban et al. 1974; Lane 1968).

Several mitochondrial functions that were totally unaffected by the moderate vitamin A supplementation in the diet, such as oxygen consumption in state 3 with five different substrates, were strikingly depressed after combination of ethanol and A supplementation; the alterations after high A and ethanol appeared much more impressive than the mere additive effect of the toxicity of high A or ethanol alone. It may be particularly significant that the depression of palmitate and palmitoyl-CoA oxidation was greater after vitamin A supplementation than after vitamin A alone. This may have contributed to the trend toward greater lipid accumulation observed in the animals fed ethanol with A supplementation compared with those given ethanol alone. It is noteworthy that vitamin A supplementation in the amount used in the present study did not result in increased lipids, contrasting with the effects of massive vitamin A overdose which causes steatosis (Singh and Singh 1978). It is also of interest that the in vitro mitochondrial studies were carried out on a mitochondrial subpopulation that presumably represents the healthiest elements. Nevertheless, the impairment of respiration and enzyme activities was striking. It is reasonable to assume that if the total mitochondrial population could have been collected, an even more profound impairment might have been found, since the ultrastructural studies revealed that some mitochondria had undergone damage so severe that they might have lost all significant functioning.

The mechanism of the potentiation of the vitamin A toxicity by ethanol consumption has not been established. It appears that the concentration of vitamin A in the liver, by itself, may not be the direct etiologic factor because ethanol treatment, in fact, resulted in a decreased level of vitamin A in the liver. Indeed, even after administration of a high vitamin A diet, ethanol-treated animals did not have an increased vitamin A expressed per 100 g BW; expressed per gram liver vitamin A concentration was even lower. It is therefore possible that as in the case of other hepatotoxic agents, the toxicity of vitamin A may be related to a metabolite, the production of which might be increased after ethanol consumption. Such a mechanism has been clearly established in the case of acetaminophen and carbon tetrachloride poisoning (Hasumura et al. 1974; Sato et al. 1981). In addition to the lowering of the vitamin A level in the liver after alcohol, indirect evidence in favor of such a possibility is provided by our preliminary results, which indicate an enhanced vitamin A catabolism in liver microsomes after chronic ethanol consumption (Sato and Lieber 1980); such enhanced catabolism may contribute also to the lowering of hepatic vitamin A, which could not be explained wholly on the basis of malabsorption (Sato and Lieber in press). It is also noteworthy that acute ethanol administration does not affect serum vitamin A, retinol binding protein, and prealbumin levels (Russell et al. 1978).

Whatever its mechanism, the potentiation of the hepatotoxicity of vitamin A by alcohol feeding has some obvious practical clinical implications. It is evident that amounts of vitamin A considered safe in normal individuals may have adverse side effects in alcoholics. Early detection of such toxicity, however, may be difficult. Indeed, with the amount of vitamin A supplementation used in our studies, serum vitamin A levels were unaffected in either the presence or the absence of ethanol (table 1). This finding contrasted with previous observations of toxicity reported after a large excess of vitamin A; Smith and

Goodman (1971) reported three cases that showed increases in plasma total vitamin A, predominantly due to a rise in retinol ester. In a fourth case, total serum carotenoids were twice the upper limits of normal (Farrell et al. 1977); in a fifth case, serum of vitamin A was slightly elevated (Russell et al. 1974). In humans, concentrations of plasma retinol-binding protein and prealbumin were unaffected (Russell et al. 1974). Similarly, retinol-binding protein was not altered in our rats fed the higher vitamin A diet. Thus, our results indicate that even in the presence of normal blood vitamin A, hepatotoxicity of vitamin A cannot be ruled out, at least not after chronic alcohol consumption. In any event, a reevaluation of the safe therapeutic dose of vitamin A in the alcoholic is required, and controlled clinical studies to settle this therapeutic issue appear warranted.

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Wharton, D.C., and Tzagoloff, A. Cytochrome oxidase from beef heart mitochondria. In: Estabrook, R.W., and Pullman, M.E., eds. *Methods in Enzymology*. Vol. X. New York: Academic Press 1967. pp. 245-250.

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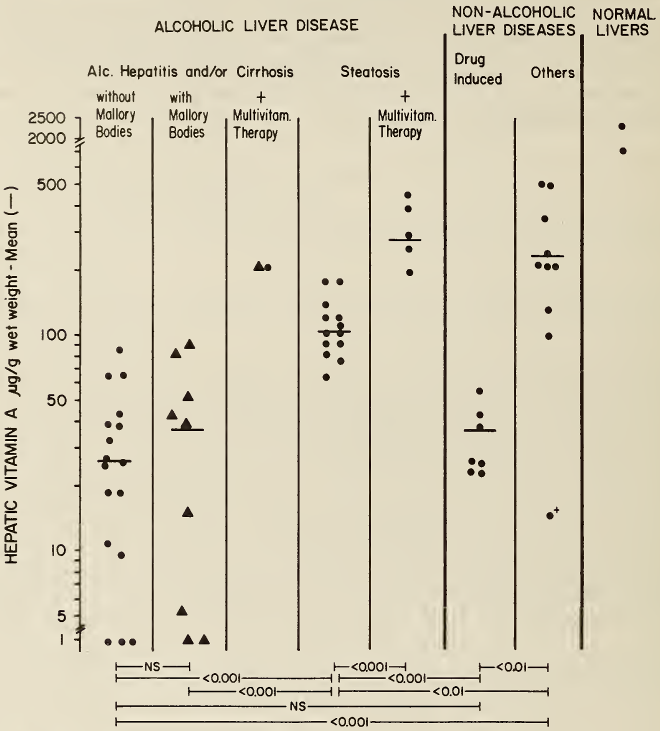
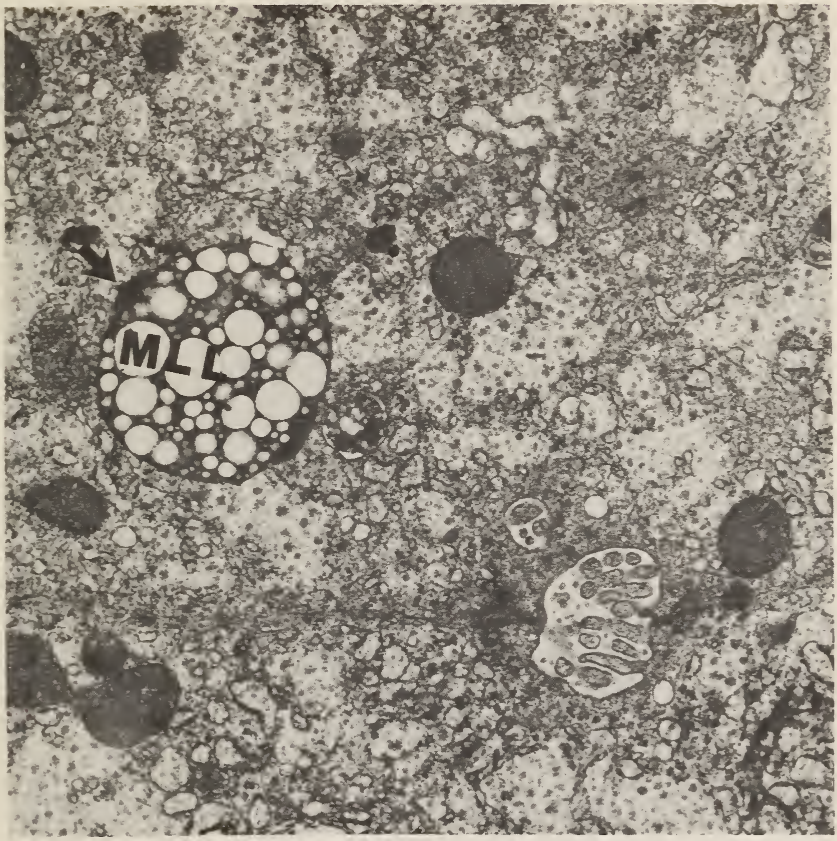
Figure 1. Hepatic Vitamin A Concentration and Mallory Bodies (▲) in Patients with Various Liver Diseases



⁺Malabsorption syndrome (not included in the mean)

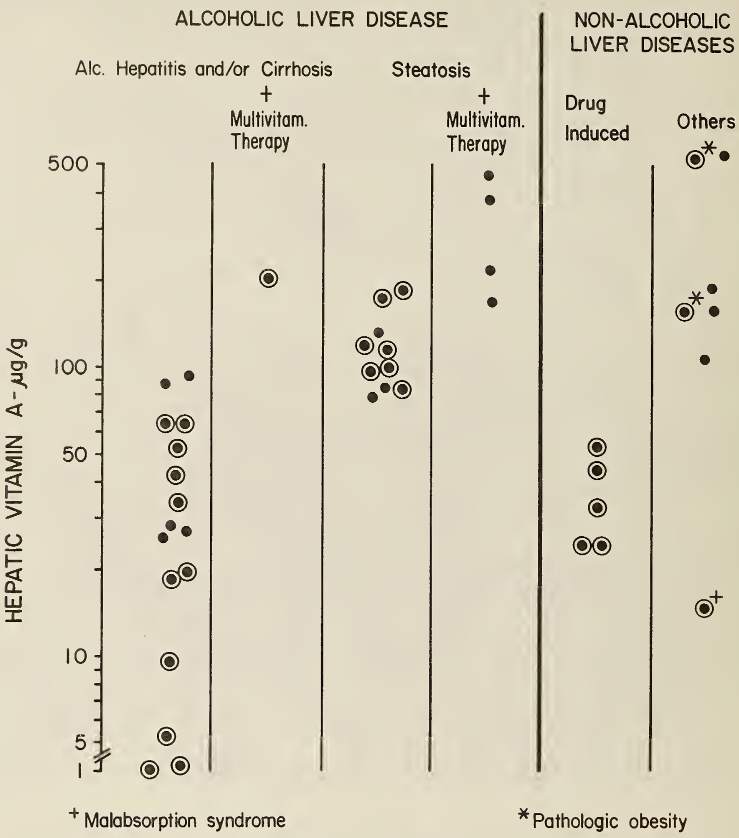
Hepatic vitamin A levels were commonly decreased particularly in patients with severe alcoholic liver disease, but there was no obvious relationship between very low levels of vitamin A and the presence of Mallory bodies.
• = Vitamin A
▲ = Mallory bodies

Figure 2. Human Hepatocyte



One multivesicular lipid-containing lysosome (MLL) is present in the pericanalicular area (bc), with a characteristic crescent (arrows) (X30,000).

Figure 3. Hepatic Vitamin A and Multivesicular Lysosomes (•) in Various Liver Diseases

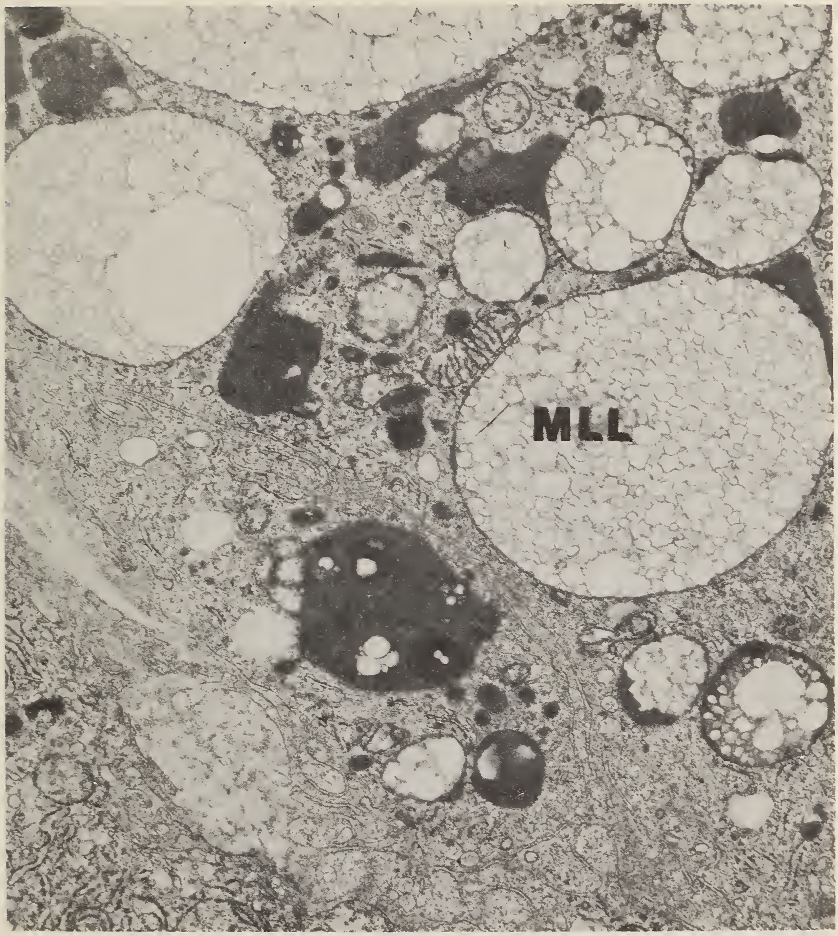


Multivesicular lipid-containing lysosomes were seen in various types of liver disease in which lowered vitamin A also was observed (see figures 2 and 4).

● = Vitamin A

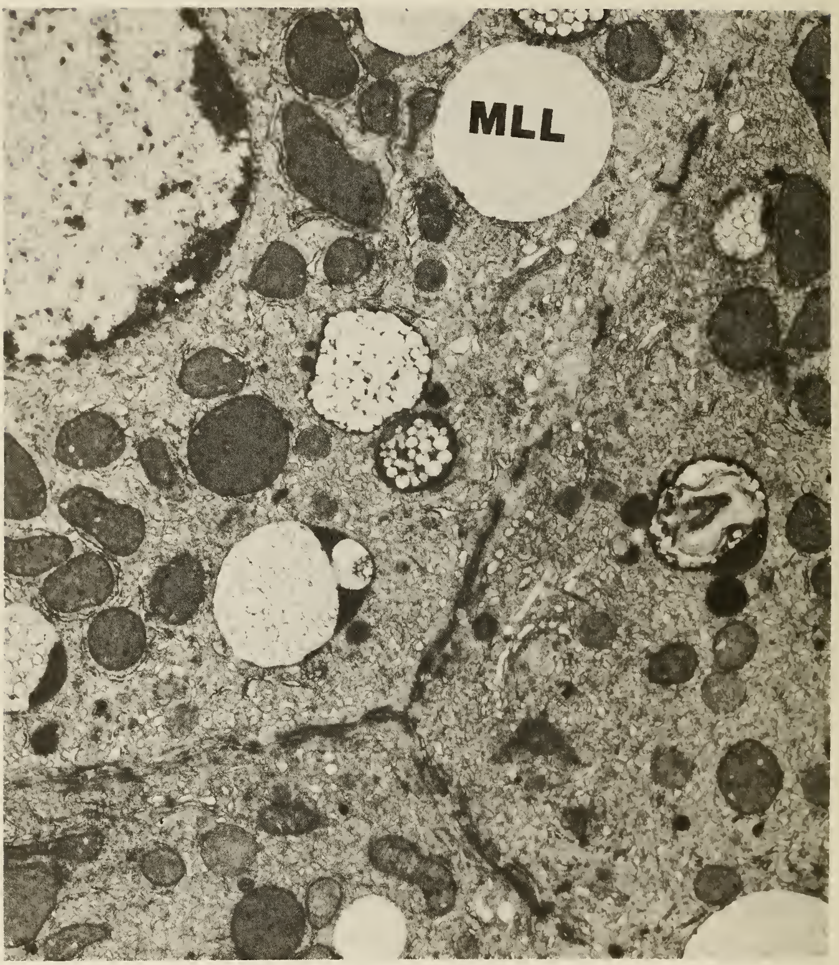
◉ = Multivesicular lysosomes

Figure 4. Human Liver



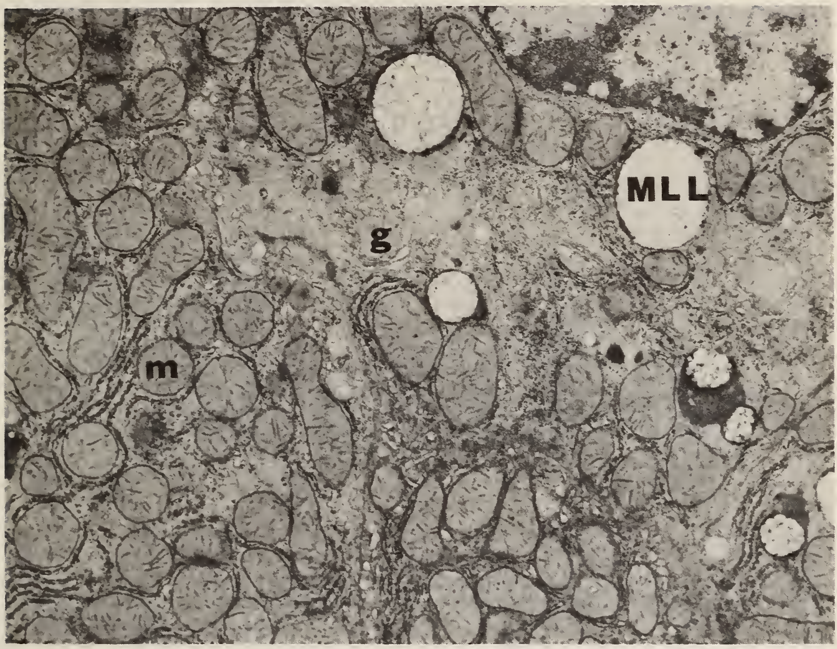
Macrophage with multivesicular lipid-containing lysosomes (MLL) (X12,000).

Figure 5. Low Vitamin A and Ethanol Rat



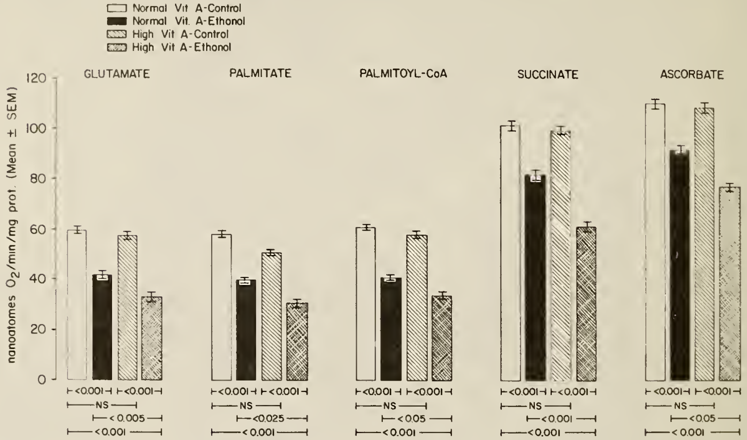
Numerous multivesicular lipid-containing lysosomes (MLL) in a hepatocyte. The lesions are more prominent than after low vitamin A control diet (figure 6); bc = equal bile ductule; g = Golgi complex (X12,000).

Figure 6. Low Vitamin A Control Rat



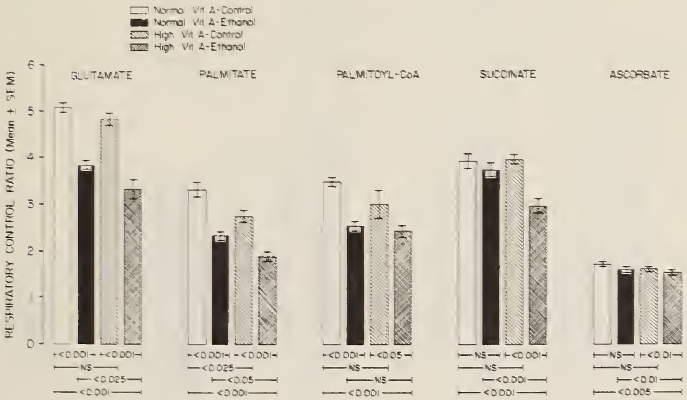
Showing few multivesicular lipid-containing lysosomes (MLL) in the absence of fat (X12,000).

Figure 7. Effect of High Vitamin A and/or Chronic Ethanol Feeding on Oxygen Consumption of Isolated Mitochondria in State 3 Respiration



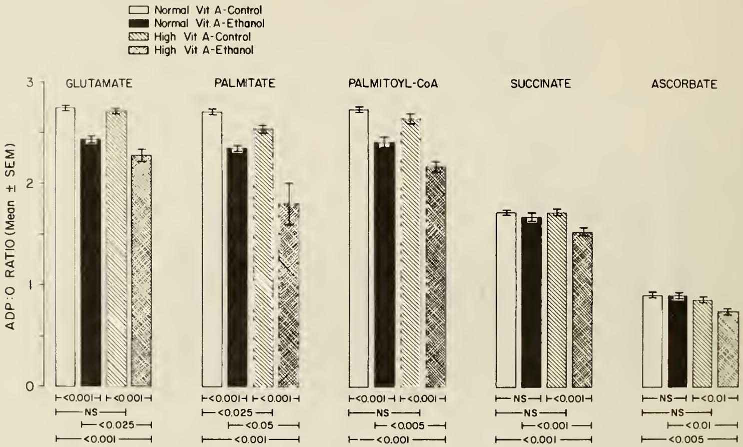
High vitamin A by itself had no effect, whereas feeding of ethanol with a normal diet resulted in a depression of the oxygen consumption with all substrates; this effect was potentiated by high vitamin A. For each group the values are given as means ± SEM of 11 pairs of animals. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 8. Effect of High Vitamin A and/or Chronic Ethanol Feeding on Respiratory Control Ratios in Isolated Mitochondria Incubated with Various Substrates



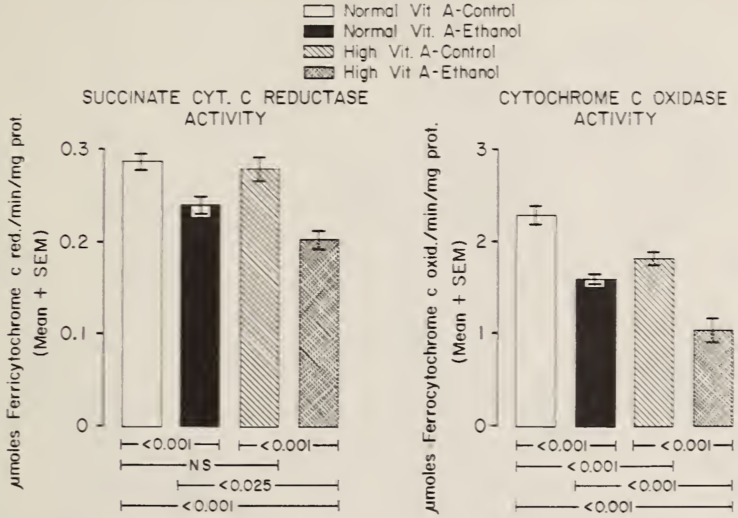
Whereas high vitamin A by itself had no effect (except for palmitate), feeding of ethanol with a normal diet resulted in a depression of the respiratory control ratios with all substrates. Except for palmitoyl-CoA, this effect was potentiated by high vitamin A. For each group the values are given as means ± SEM of 11 pairs of animals. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 9. Effect of High Vitamin A and/or Ethanol Feeding on ADP:O Ratios in Isolated Mitochondria Incubated with Various Substrates



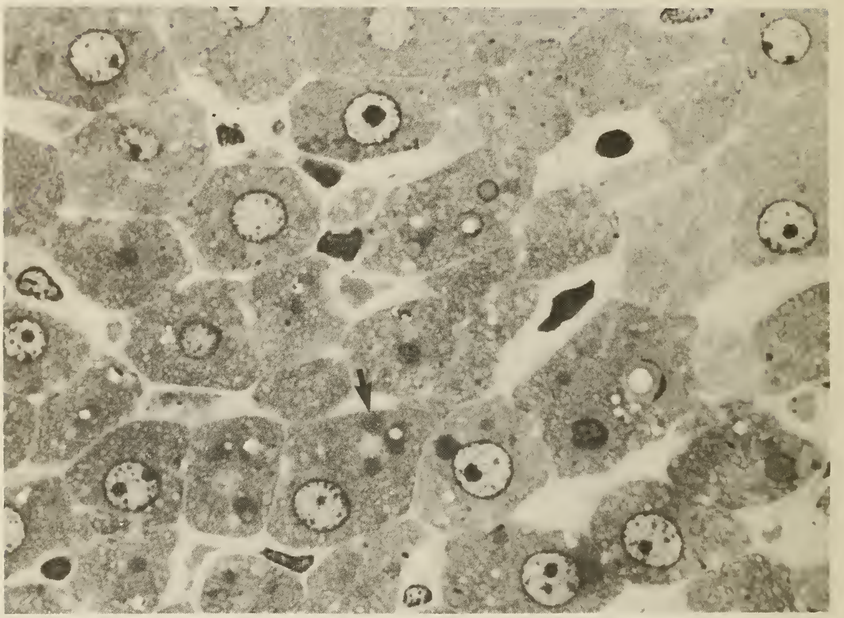
Whereas high vitamin A by itself had no effect (except for a palmitate), feeding of ethanol with a normal diet resulted in a depression of the ratios with glutamate, palmitate, and palmitoyl-CoA; this effect was potentiated by the high vitamin A diet. For each group the values are given as means ± SEM of 11 pairs of animals. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 10. Effect of High Vitamin A and/or Chronic Ethanol Consumption on Liver Mitochondrial Succinate Cytochrome C Reductase and Cytochrome C Oxidase Activities



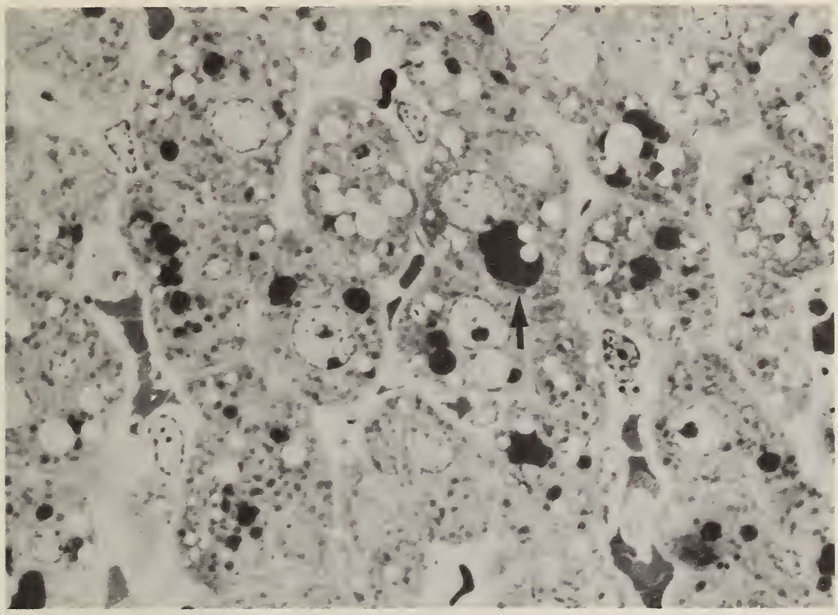
For each group the values are given as means ± SEM of 11 pairs of animals. Whereas vitamin A by itself depressed only cytochrome c oxidase activity, both enzymes were affected by ethanol, and the reduction was most pronounced when ethanol was given with the high vitamin A diet. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 11a. Liver of Rat Fed Normal A and Ethanol Diet



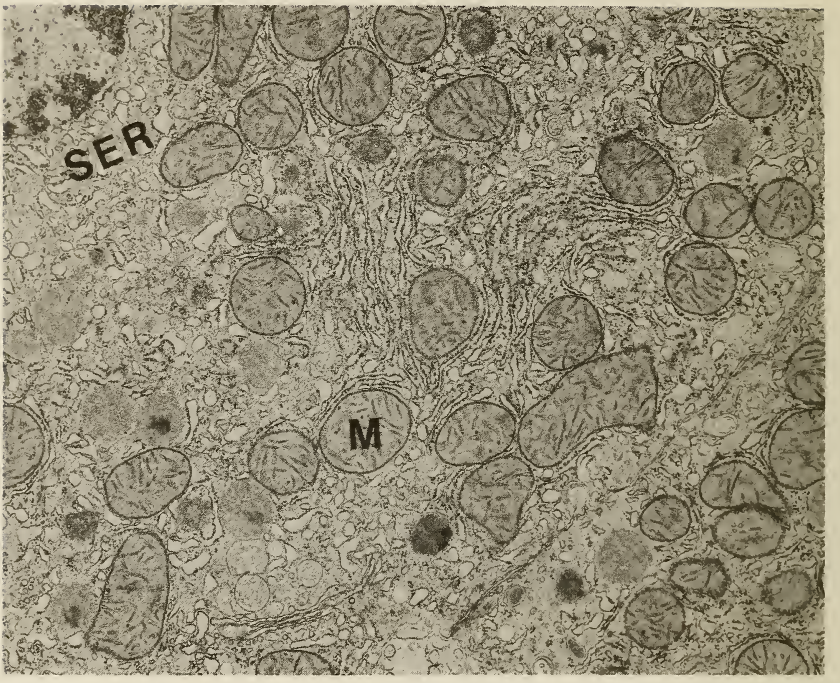
Toluidine-blue stain (X1,000). Epon thick section (viewed by light microscopy) shows a few enlarged mitochondria (arrows). Some fat droplets are also present. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 11b. Liver of Rat Fed High A Ethanol Diet



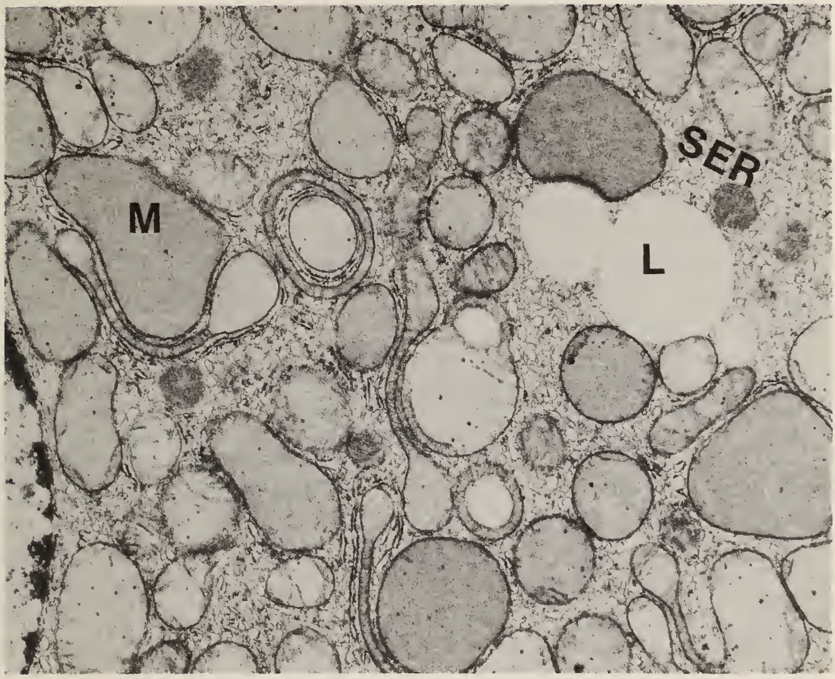
Toluidine-blue stain (X1,000). Epon thick section shows irregularly shaped megamitochondria, one of which (arrow) has the same size as the nucleus; numerous enlarged mitochondria are also present in other hepatocytes. Accumulation of fat is more pronounced than in rats fed a normal A and ethanol diet. (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 12a. Normal A Control Rat



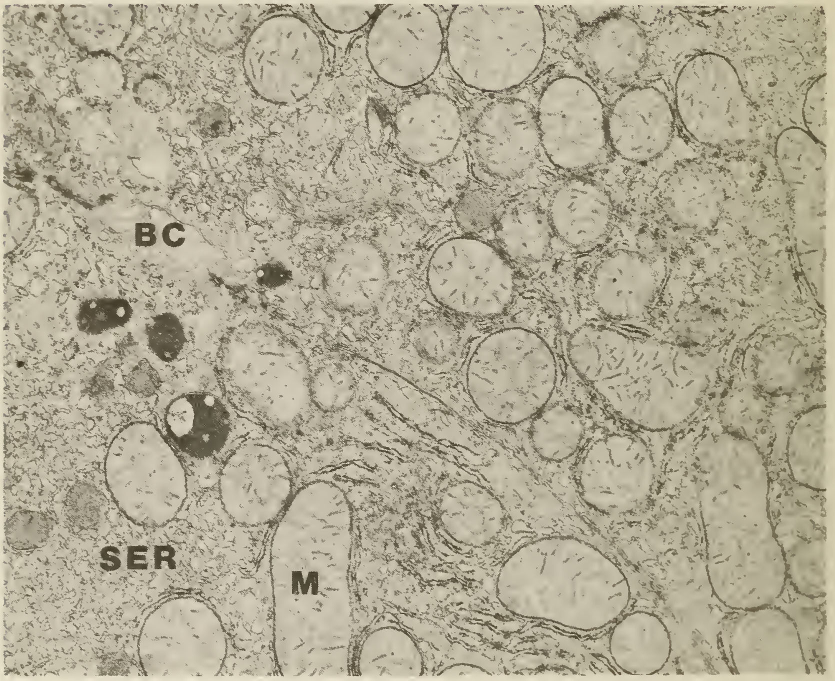
Organelles are normal. M = mitochondrion. SER = smooth endoplasmic reticulum. Electron micrograph of pericentral hepatocytes (X18,000). (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 12b. Electron Micrographs of Pericentral Hepatocytes, Normal A Ethanol Rat



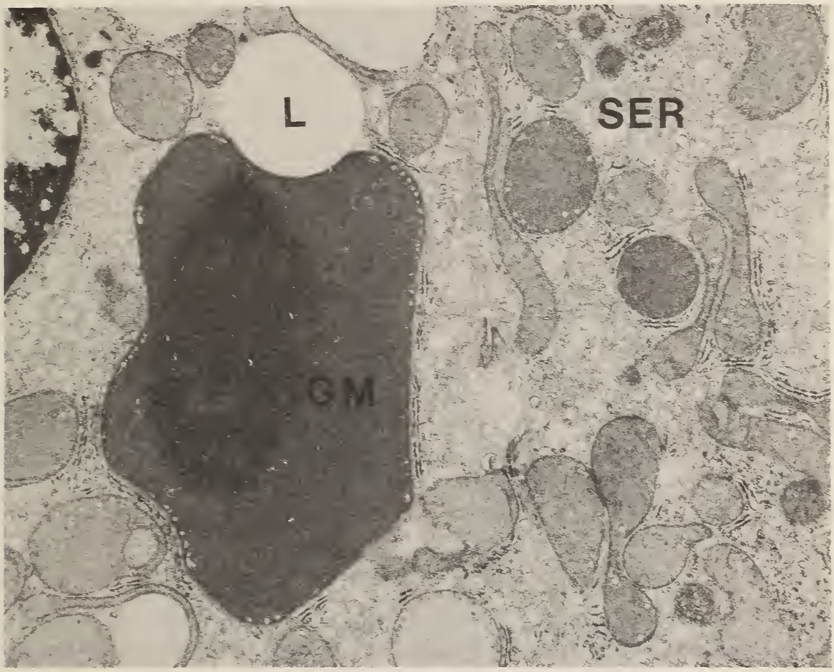
Some mitochondria are enlarged (M). The cristae are rudimentary or have virtually disappeared and are replaced by a dense matrix. SER = proliferated smooth endoplasmic reticulum. L = lipid droplet (X18,000). (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 13a. Electron Micrograph of Pericentral Hepatocytes, High A Control Rat



Electron Micrograph of Pericentral Hepatocytes; M = mitochondria showing slight swelling and clear matrix; SER = proliferated smooth endoplasmic reticulum (X18,000). (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Figure 13b. Electron Micrograph of Pericentral Hepatocytes, High A and Ethanol Rat



Giant mitochondrion (GM) approaches the size of a nucleus. It contains an unusual dense matrix with a large fusiform crystalline inclusion. This and adjacent mitochondria display severe disorganization of the cristae. L = lipid droplet; SER = proliferated smooth endoplasmic reticulum (X18,000). (From Leo, M.A.; Arai, M.; Sato, M.; and Lieber, C.S. Hepatotoxicity of vitamin A and ethanol in the rat. *Gastroenterology*, 1982. Copyright 1982 by Elsevier North-Holland, Inc. [NY].)

Table 1. **Hepatic Vitamin A and Serum Retinol-Binding Protein (RBP), Prealbumin (PA), and Vitamin A in Patients with Alcoholic and Nonalcoholic Liver Diseases**

Disease		Liver	Serum		
		Vitamin A	Vitamin A	RBP	PA
		μg/wet wt.	μg/100ml	μg/ml	μg/ml
Alcoholic liver disease					
Alcoholic hepatitis and/or cirrhosis	(13)	42.2 ± 7.2[*]	22.4 ± 4.5[**]	30.4 ± 6.5[***]	119.5 ± 26.5[**]
Steatosis	(10)	98.3 ± 8.2	42.6 ± 6.3	55.3 ± 8.8	258.7 ± 47.0
Steatosis + multivitamin therapy	(4)	324.1 ± 58.6[****]	60.4 ± 11.9	65.2 ± 11.3	280.0 ± 67.0
Nonalcoholic liver diseases					
Drug-induced	(3)	36.0 ± 2.6[†]	37.1 ± 1.7	40.8 ± 4.9	183.0 ± 33.0
Others	(5)	259.7 ± 92.3	41.9 ± 2.7	38.6 ± 9.4	184.0 ± 48.0

[*] $p<0.001$. [**] $p<0.02$. [***] $p<0.05$. [****] $p<0.001$ when compared to the steatosis group.

[†] $p<0.001$ when compared to others.

Table 2. **Hepatic and Serum Vitamin A and Serum Retinol-Binding Protein (RBP) in Rats Fed a Normal or a Low Vitamin A Diet With or Without Ethanol**

Diet		Liver Vit. A μg/g	Serum Vit. A μg/100 ml	RBP μg/ml
Normal vitamin A*				
Ethanol	(5)	114.1 ± 10.5	42.5 ± 2.9	51.6 ± 3.2
Pair-fed controls	(5)	288.6 ± 27.4	42.3 ± 2.8	48.8 ± 3.2
p		<0.001	N.S.	N.S.
Low vitamin A**				
Ethanol	(9)	0	10.2 ± 2.9	12.4 ± 4.1
Pair-fed controls	(9)	0.04 ± 0.03	9.4 ± 3.1	8.0 ± 3.4
p		N.S.	N.S.	N.S.

Note: Values are given as means ± SEM.

*Average daily intake was 350 IU.

**Average daily intake was 1 IU.

Table 3. **Serum and Liver Vitamin A in Rats Fed a Normal or a High Vitamin A Diet With or Without Ethanol**

Diet	Serum Vitamin A μg/100ml	Liver Vitamin A μg/g	 μg/100g B.W.
Normal vitamin A			
Ethanol-fed rats (5)	42.5 ± 2.8	119.7 ± 11*	421 ± 43*
Pair-fed controls (5)	42.3 ± 2.8	302.7 ± 29**	799 ± 43
p	N.S.	<0.01	<0.01
High vitamin A			
Ethanol-fed rats (12)	39.8 ± 1.8	235.0 ± 16	907 ± 63
Pair-fed controls (12)	40.7 ± 2.4	636.2 ± 40	1809 ± 107
p	N.S.	<0.001	<0.001

Notes: Rats (number shown in parentheses) were fed diets with normal or high vitamin A content (with or without ethanol) for 8 weeks. Serum and liver vitamin A were measured as described under "Methods." The values are given as means ± SEM.

*$p<0.001$. **$p<0.05$ when compared to high vitamin A and ethanol.

Table 4. Effect of High Vitamin A and/or Ethanol Consumption on Liver Mitochondrial Enzyme Activities

Diet		NADH Dehydrogenase U*/mg Protein	Succinate Dehydrogenase U**/mg Protein	NADH-Cytochrome c Reductase U***/mg Protein	β-Hydroxybutyrate Cytochrome c Reductase U***/mg Protein
Normal vitamin A					
Ethanol-fed rats	(11)	3.34 ± 0.16	0.186 ± 0.008	0.515 ± 0.027	0.334 ± 0.015
Pair-fed controls	(11)	3.94 ± 0.14	0.205 ± 0.007	0.699 ± 0.029	0.420 ± 0.013
p		<0.01	N.S.	<0.005	<0.001
Normal vitamin A					
Ethanol-fed rats	(11)	3.16 ± 0.21	0.162 ± 0.007	0.499 ± 0.036	0.295 ± 0.017
Pair-fed controls	(11)	3.84 ± 0.17	0.201 ± 0.007	0.629 ± 0.022	0.393 ± 0.017
p		<0.025	<0.001	<0.01	<0.001

Notes: Rats (number shown in parentheses) were fed diets with normal or high vitamin A content (with or without ethanol) for 8 weeks, and mitochondria were prepared and studied as described under "Methods." The values are given as means ± SEM.
U^{*} = μmoles ferricyanide reduced/min; U^{**} = μmoles succinate oxidized/min.; U^{***} = μmoles ferrcytochrome c reduced/min.

Table 5. Effect of High Vitamin A and/or Ethanol Consumption on Liver Glutamate Dehydrogenase (GDH) Activity

Diet		Mitochondrial GDH	Total Liver GDH	
		U/mg Protein[1]	U/g	U/100g B.W.
Normal vitamin A				
Ethanol-fed rats	(11)	1.6 ± 0.08	150.2 ± 7.2	338.9 ± 27.5
Pair-fed controls	(11)	2.0 ± 0.12[*]	178.1 ± 14.2[**]	474.7 ± 57.7[**]
p		<0.01	N.S.	N.S.
High vitamin A				
Ethanol-fed rats	(11)	1.4 ± 0.07	141.0 ± 9.6	310.2 ± 13.5
Pair-fed controls	(11)	1.8 ± 0.07	176.3 ± 8.7	465.1 ± 23.1
p		<0.001	<0.025	<0.001

Notes: Rats (number shown in parentheses) were fed diets with normal or high vitamin A content (with or without ethanol) for 8 weeks. Serum GDH activity was measured as described under "Methods." The values are given as means ± SEM.

[1] U = μmoles NADH oxidized/min.

[*] $p<0.001$; [**] $p<0.05$ when compared to high A and ethanol.

Table 6. Effect of High Vitamin A and/or Ethanol Consumption on Cytochrome Content in Rat Liver Mitochondria

Diet		Cytochrome $c+c_1$	Cytochrome b	Cytochrome aa_3
		nmoles/mg Protein	nmoles/mg Protein	nmoles/mg Protein
Normal vitamin A				
Ethanol-fed rats	(11)	0.293 ± 0.013[*]	0.213 ± 0.007[**]	0.137 ± 0.011[***]
Pair-fed controls	(11)	0.312 ± 0.001[¶]	0.255 ± 0.008[¶¶]	0.214 ± 0.006
p		N.S.	<0.001	<0.001
High vitamin A				
Ethanol-fed rats	(11)	0.254 ± 0.013	0.176 ± 0.011	0.102 ± 0.007
Pair-fed controls	(11)	0.294 ± 0.013[†]	0.223 ± 0.011[†]	0.173 ± 0.013[††]
p		<0.05	<0.025	<0.001

Notes: Rats (number shown in parentheses) were fed diets with normal or high vitamin A content (with or without ethanol) for 8 weeks. Mitochondria were prepared and cytochrome content was measured as described under "Methods." Values are the means ± SEM.

[*] $p<0.05$; [**] $p<0.01$; [***] $p<0.025$ when the values are compared with high A and ethanol rats.

[¶] $p<0.005$; [¶¶] $p<0.001$ when compared with high vitamin A and ethanol rats.

[†] N.S.; [††] $p<0.025$ compared with normal A control group.

SESSION IV: ALCOHOL METABOLISM

The Hepatic Metabolic Pathways of Ethanol Oxidation

Ellen R. Gordon

Introduction

There are three metabolic pathways within the liver by which ethanol can be oxidized (Lieber 1977; Plapp 1975). Although the characteristics of each system have been well documented, their relative contribution to the disposition of ethanol under a variety of conditions has, as yet, not been completely established.

Traditionally, it has been considered that the main pathway for ethanol oxidation involves alcohol dehydrogenase (ADH), an enzyme of the cytosol. In this pathway, the hydrogen 1-R of ethanol is transferred to the cofactor nicotinamide adenine dinucleotide (NAD^+), which is then converted to its reduced form (NADH). Measurements of alcohol dehydrogenase activity in rat liver homogenates have yielded a maximum rate of 300 μmole/g wet wt/hr in both fed and starved animals, which is equal to or greater than the average rate of ethanol utilization in vivo (Crow et al. 1977; Plapp 1975). Thus, under optimal conditions (i.e., high NAD^+ and ethanol concentrations and low NADH and acetaldehyde concentrations), the activity of alcohol dehydrogenase is more than sufficient to account for observed in vivo rates of ethanol metabolism. However, conditions are rarely optimal in the cell. The reported "Km's" of ethanol for alcohol dehydrogenase range from 0.2 to 2.0 mM. In fact, at saturating ethanol concentrations (5 to 10 mM) and low acetaldehyde levels, the rate of ethanol oxidation will depend on the relative levels of free NAD^+ and NADH. In vitro experiments indicate that the dissociation of the enzyme-NADH complex is the rate-limiting step in this reaction (Theorell and Chance 1951). In vivo, this in turn is governed by the cell's capacity to handle NADH (Israel et al. 1979). However, the metabolic control of reducing equivalents is complex. Therefore, this explanation is undoubtedly an oversimplication of the in vivo factors that regulate ethanol metabolism. Therefore, the rate of ethanol oxidation in the ADH pathway is governed not only by the level of the enzyme but by the cell's capacity to supply the cofactors and handle the products formed, so that any quantitative comparison of enzyme activities with in vivo rates of ethanol elimination (as has been proposed by Crow et al. [1977]) is exceedingly naive.

NOTE: Figures and tables appear at end of paper.

Recently, it has been demonstrated that human and monkey livers contain multiple molecular forms of ADH, with the number and amounts of each form varying depending on the nutritional and genetic background of the donor (Sytowski and Vallee 1979). One of these forms, NADH^2, exhibits kinetic properties that differ markedly from the other isoenzymes (Dafeldecker et al. 1981; Li and Magnes 1975). Its Km value is in the mM range, and unlike the other forms of ADH it is remarkably insensitive to pyrazole and 4-methyl pyrazole. NADH^2 has not yet been detected in hepatic preparations from rodents or baboons, and at this time it is difficult to make a precise statement concerning the importance of its role in ethanol oxidation.

The first indication of an interaction of ethanol with the microsomal fraction of the hepatocyte was provided by the morphological observation that in rats ethanol feeding results in a proliferation of the smooth endoplasmic reticulum (Iseri et al. 1966). This response was similar to reactions observed after the administration of a wide variety of xenobiotic compounds, including known hepatotoxins (Conney 1967; Meldolesi 1967) and food additives (Lane and Lieber 1967). It is perhaps significant that most of these compounds are also metabolized to some extent in the smooth endoplasmic reticulum. Orme-Johnson and Zeigler (1965) described a microsomal system capable of methanol and ethanol oxidation. Zeigler and Mitchell (1972) characterized this system further and noted that it could not oxidize long-chain aliphatic alcohols, such as butanol or n-propanol, and that it was sensitive to catalase inhibitors, azide and cyanide. It was concluded that this microsomal system oxidized ethanol through a H_2O_2-mediated reaction involving catalase, a contaminate of the microsomal preparations.

A microsomal-ethanol oxidizing system (MEOS) distinct from catalase has been characterized (Lieber and DeCarli 1968, 1973; Ohnishi and Lieber 1977; Teschke et al. 1976, 1977). The system requires NADPH and O_2 and is relatively insensitive to catalase inhibitors, and it exhibits an ability to oxidize long-chain aliphatic alcohols that are not substrates for catalase. For ethanol, the Km of MEOS differs markedly from that of ADH (being 8 to 10 mM), as does its in vitro pH optimum (7.4 versus 10), as well as its cofactor requirements (figure 1 and table 1).

The third metabolic pathway in which ethanol can be oxidized is a peroxidative pathway involving catalase. Since catalase, a peroxisomal enzyme in the presence of H_2O_2, can oxidize ethanol in vitro, and because the maximal activity of catalase in the liver is very high, it was considered by some investigators that this system accounted for the non-ADH oxidation of ethanol (Thurman et al. 1972, 1976). However, it is now generally accepted that this pathway is limited by the rate of H_2O_2 generation rather than the amount of catalase itself

(Bartlett 1952; Feytman and Leighton 1973). Thus, an indirect assessment of the importance of catalase in ethanol metabolism can be derived from the rate of H_2O_2 generation in the liver. The physiological rate of H_2O_2 is low (3.6 μmol/hr/g of liver) (Boveris et al. 1972) and is equivalent to only 2 percent of the in vivo rate of ethanol oxidation (178 μmol/hr/g of liver) (Lieber and DeCarli 1972). Some in vivo experiments, although not completely conclusive, also support this conclusion. The rate of ethanol metabolism in rats is virtually unaffected by treatment with 3 amino-1,2,4 triazole (AT), even though it caused a 90 percent inhibition of hepatic catalase (Kinard et al. 1956). In a similar study, Smith (1961) demonstrated that in rats pretreated with AT, methanol oxidation was decreased by 70 percent, although ethanol oxidation was unaffected.

Relative Contribution of the Three Metabolic Pathways to the Oxidation of Ethanol

Despite the considerable controversy that existed for many years, it is now generally accepted that under normal circumstances H_2O_2-mediated ethanol oxidation by catalase contributes little to the oxidation of ethanol within the liver. However, the relative importance of the alcohol dehydrogenase and the microsomal alcohol oxidizing pathways in the metabolism of ethanol remains unsettled. Several approaches have been used to determine the contribution of the non-ADH pathway. These include (a) an examination of the incomplete inhibition of ethanol metabolism in the presence of ADH inhibitors such as pyrazole and 4-methyl pyrazole (Grunnet et al. 1973; Higgins 1979; Matsuzaki et al. 1977); (b) an observation of the pattern of labeling of glucose and lactic acid, as derived from stereo-specific labeled ethanol (Harve et al. 1977; Rognstad 1974); (c) an examination of the increased rate of ethanol metabolism at high ethanol concentrations well above those needed to saturate alcohol dehydrogenase (Hawkins et al. 1966; Korsten et al. 1975; Lieber and DeCarli 1970; Matsuzaki et al. 1981; Pikkarainen and Lieber 1980); (d) the observations that the metabolic changes arising from the oxidations of ethanol in the ADH pathway are attenuated in the normal animal at high ethanol levels (Guynn and Pieklik 1975) and in animals consuming ethanol for prolonged periods (Salaspuro et al. 1981); and (e) an examination of the metabolism of ethanol in mice deficient in ADH (Burnett and Felder 1980).

Table 2 lists inhibitors used to estimate the contribution of the non-ADH pathway to the metabolism of ethanol. These studies support the concept that pathways other than ADH participate in the metabolism of ethanol. However, the pyrazoles compete with ethanol for alcohol dehydrogenase, and the degree of inhibition will depend upon the ethanol concentration (Higgins 1979). A sufficiently high level, therefore, must be used to completely inhibit ADH; unfortunately, however, even at low levels of pyrazole or 4-methyl pyrazole, these inhibitors also reduce the activity of the microsomal ethanol oxidizing system (Teschke et al. 1977). Therefore, quantitative estimation of the contribution of the non-ADH pathway in the oxidation of ethanol is difficult using this approach.

The fate of I-R-H^3 ethanol, which produces a 4 A NAD^{3}H in the alcohol dehydrogenase reaction, has been compared with that of other substrates of cytosolic A-type NAD-linked dehydrogenase as a means of evaluating the relative roles of ADH and non-ADH pathways. The NAD^{3}H produced in this reaction then labels products of various other dehydrogenase systems, especially glucose (Rognstad 1974). Tritiated water is also formed. On the other hand, tritiated water is the only initial product formed if 1-R-^{3}H ethanol is oxidized by either catalase or MEOS. Therefore, if a non-ADH pathway participates in ethanol oxidation, there would be a relatively higher water/glucose ratio for the tritium obtained from 1-R-H^3 ethanol compared to that from 2^{3}H lactate. Using these procedures in conjunction with pyrazole, Rognstad (1974) noted that approximately 30 percent of the ethanol oxidized could be accounted for by non-ADH pathway. In a similar group of experiments, Havre and his colleagues (1977) estimated that about 90 percent of ethanol oxidation occurred in the ADH pathway. The reason for this apparent discrepancy has not been clarified. However, both these investigators used pyrazole, an ADH inhibitor, which as noted also inactivates the MEOS pathway. Further studies using this approach without inhibitors would aid in determining the relative roles of these pathways in the oxidation of ethanol.

Recent in vitro studies using isolated hepatocytes and in vivo studies using baboons support the concept that the rate of ethanol oxidation is dose-dependent at ethanol concentrations greater than those required to saturate ADH. In the study conducted in vivo (Pikkarainen and Lieber 1980), ethanol elimination rates were measured in seven baboons fed alcohol for 2 to 7 years and their pair-fed controls. Using a constant ethanol infusion to maintain blood ethanol levels at three different levels (5, 10, and 50 mM), ethanol elimination rates were found to be faster when blood ethanol levels were maintained at 50 mM than at 10 or 5 mM (figure 2 and table 3). These differences could not be accounted for by extrahepatic losses. The results of these in vivo experiments indicate that the rate of ethanol oxidation is concentration-dependent and cannot be accounted for by a low K_m alcohol dehydrogenase. No high K_m alcohol dehydrogenase

(ADH) has as yet been detected in baboon liver. Therefore, the most likely explanation is a non-ADH oxidation of ethanol with the participation of MEOS which has a K_m of about 10 mM, and has been shown to be "inducible" by the chronic intake of ethanol.

Utilizing a different approach, it has been demonstrated in vitro that the rates of ethanol oxidation in isolated rat hepatocytes increased with increasing ethanol concentrations even at near saturation of the ADH pathway (table 4). After inhibition of ADH by pyrazole (2 mM), approximately 30 percent of ethanol-oxidizing activity remained, and this activity of the non-ADH pathway was also dependent upon ethanol concentration. The apparent K_m of the non-ADH pathway for ethanol was 13 mM, which is comparable to the value for MEOS. Inhibition of catalase by azide affected the activity of the non-ADH pathway by less than 10 percent. After chronic ethanol feeding, the rates of ethanol oxidation in isolated hepatocytes increased significantly compared to those in pair-fed controls. This adaptive increase of ethanol oxidation was more striking at a high ethanol level (30 mM) than at a low one (10 mM) (table 5). Significant differences between the two groups remained after inhibition of ADH and catalase (table 6).

As ethanol is oxidized in the ADH pathway, the redox state of the hepatocyte is shifted to a more reduced level (Lieber 1977). This change is diminished both in the naive rat at high ethanol concentrations (Guynn and Pieklek 1975), and in ethanol-fed rats (Baraona et al. 1980). Similar findings were observed in ethanol-fed baboons, which developed the full spectrum of alcoholic liver disease (Salaspuro et al. 1981). It was demonstrated that chronic ethanol feeding without nutritional deficiencies decreased the inhibitory effect of ethanol on galactose elimination in these animals. Similar findings were obtained in in vitro studies in which inhibition by ethanol of tricarboxylic acid cycle activity and fatty acid oxidation, and shifting of the redox state of the cytosol to a more reduced state, was less in liver slices prepared from ethanol-fed baboons than in pair-fed controls. The diminution of the redox changes after chronic feeding could be explained on the basis of increased NADH reoxidation or decreased ethanol oxidation by ADH or an increase in the participation of the microsomal ethanol-oxidizing pathway.

Perhaps the most convincing evidence for the participation of a non-ADH pathway in the disposition of ethanol comes from data obtained by a somewhat different approach. A species of mice devoid of ADH still have the capacity to oxidize ethanol, albeit at rates up to only two-thirds of that observed in normal mice (Burnett and Felder 1980). It is noteworthy that these mice have significant levels of MEOS.

This brief survey of the literature indicates that within the liver there are two major pathways (ADH and MEOS) by which ethanol is oxidized. The characteristics of these systems indicate that at low ethanol concentrations the ADH pathway predominates, but as ethanol levels are increased a greater proportion of ethanol is oxidized in the microsomal system. With prolonged ethanol intake it appears that a greater fraction of the ethanol is oxidized in the microsomal ethanol-oxidizing system.

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Figure 1. Methods of Ethanol Oxidation

A.

$$CH_3CH_2OH + NAD^+ \xrightarrow[\text{ADH}]{} CH_3CHO + NADH + H^+$$

B.

$$CH_3CH_2OH + NADPH + H^+ + O_2 \xrightarrow[\text{MEOS}]{} CH_3CHO + NADP^+ + 2H_2O$$

C.

$$NADPH + H^+ + O_2 \xrightarrow[\text{NADPH Oxidase}]{} NADP^+ + H_2O_2$$

$$+$$

$$H_2O_2 + CH_3CH_2OH \xrightarrow[\text{Catalase}]{} 2H_2O + CH_3CHO$$

D.

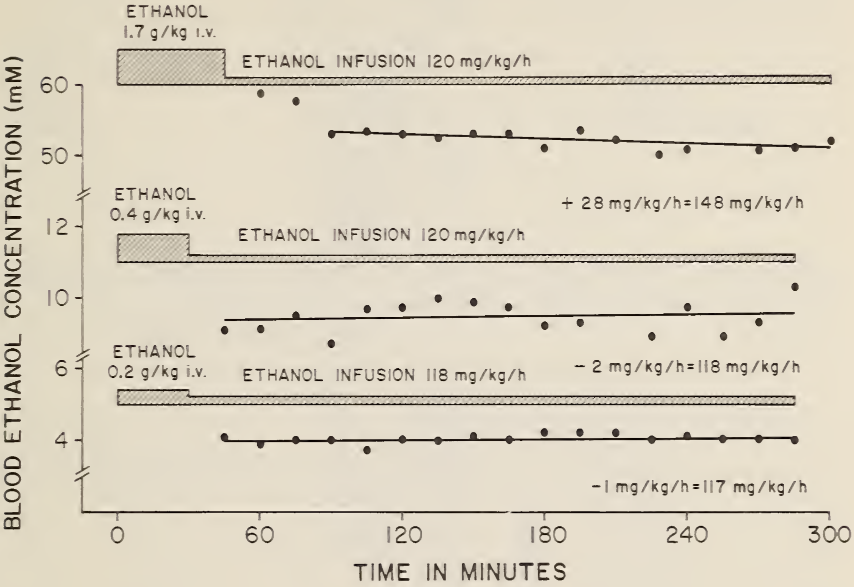
$$\text{HYPOXANTHINE} + H_2O + O_2 \xrightarrow[\text{Xanthine Oxidase}]{} \text{XANTHINE} + H_2O_2$$

$$+$$

$$H_2O_2 + CH_3CH_2OH \xrightarrow[\text{Catalase}]{} 2H_2O + CH_3CHO$$

Ethanol oxidation by (A) alcohol dehydrogenase (ADH), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide, reduced form (NIDH); (B) the hepatic microsomal ethanol-oxidizing system (MEOS), nicotinamide adenine dinucleotide phosphate (NADP); (C) a combination of NADPH oxidase and catalase; or (D) Zanthin oxidase and catalase.

Figure 2. Representative Infusion Study in a Control Baboon



Three different experiments were carried out 1 week apart. Blood ethanol levels (4-6 mM, 8-12 mM, or 40-60 mM) were maintained by constant infusion of ethanol. The rate of ethanol elimination was calculated from these data (see text).

Table 1. **Characteristics of Hepatic Alcohol Oxidizing Systems**

Enzyme	Cellular Site	Km mM	Apparent Vmax mM	Cofactors	Product	pH Optimum
ADH	Cytosol	0.2-2.6	5-10	NAD	Acetaldehyde NADH	9.6
MEOS	Microsomes	13	50-60	NADPH O_2	Acetaldehyde NADP	7.0
Catalase	Peroxisomes			H_2O_2 generating system	Acetaldehyde H_2O	

Table 2. Metabolic Inhibitors of Alcohol Oxidizing Systems

Inhibitor	Nature of Inhibition	Enzyme Systems Inhibited
Pyrazole	Competitive	ADH
4-Methyl Pyrazole	Competitive	ADH
Azide		Catalase
Cyanide		Catalase MEOS

Table 3. Elimination Rate of Ethanol in Seven Pairs of Alcohol-Fed and Control Baboons at Three Different Blood Ethanol Levels (Mean ± SEM)

	Elimination Rate of Ethanol (mg/kg/hr)		
Blood Ethanol Level (mM)	Ethanol-fed	Controls	p
4-6	132 ± 6.1*	115 ± 9.9	N.S.
	$p < 0.05$	N.S.	
8-12	148 ± 4.1	114 ± 8.2	<0.01
	$p < 0.05$	$p < 0.05$	
40-60	179 ± 10.0*	144 ± 9.8	0.05

*4-6 versus 40-60 mM; $p<0.01$.

Table 4. Effects of Ethanol Concentration and ADH Inhibition by Pyrazole (2 mM) on Rates of Ethanol Oxidation in Isolated Hepatocytes from Chow-Fed Rats

Ethanol mM	Number of Preparations	No Inhibitor	Pyrazole 2 mM
10	10	$1.51 \pm 0.09^{\dagger}$	$0.31 \pm 0.02^{\dagger}$
20	10	$2.15 \pm 0.08^{*\dagger}$	$0.69 \pm 0.03^{*\dagger}$
30	10	$2.69 \pm 0.09^{**\dagger}$	$0.84 \pm 0.05^{*\dagger}$
50	6	$3.29 \pm 0.10^{**\dagger}$	$0.99 \pm 0.04^{**\dagger}$

Note: Ethanol oxidized is expressed as µmoles/min/g wet wt. (mean ± SEM).

*, **$p<0.01$ and $p<0.001$, respectively, as determined by the comparison of the means of the results at 10 mM ethanol and at higher concentrations in each group.

†$p<0.001$ as determined by comparison of the means of the results with and without inhibitor.

Table 5. A Comparison of Rates of Ethanol Oxidation in Isolated Hepatocytes of Nine Rats Fed Ethanol Chronically and Pair-Fed Controls

	Rates of Ethanol Oxidation	
	µmoles/g/wet	wt/min
Ethanol mM	Ethanol-fed	Controls
10	1.38 ± 0.07	$1.10 \pm 0.03^{*}$
20	2.18 ± 0.08	$1.81 \pm 0.05^{*}$
40	2.92 ± 0.22	2.10 ± 0.10

Note: Ethanol oxidized is expressed as µmoles/min/g wet wt. (mean ± SEM).

*$p<0.01$ as determined by comparison of the means of the values between the groups at each ethanol concentration.

Table 6. **Rates of Ethanol Oxidation in Isolated Hepatocytes of Five Rats Fed Ethanol Chronically and of Pair-Fed Controls in the Absence and Presence of Pyrazole (2 mM) and Azide (1 mM)**

	Ethanol Concentrations		
Treatment	10 mM	30 mM	p
Ethanol-fed rats			
No inhibitor (A)	1.94 ± 0.09	2.62 ± 0.07	0.001
Pyrazole and azide (B)	0.47 ± 0.03	1.03 ± 0.05	0.01
Controls			
No inhibitor (A)	1.47 ± 0.04	1.79 ± 0.03	0.01
Pyrazole and azide (B)	0.37 ± 0.02	0.63 ± 0.03	0.05
p^A	< 0.05	< 0.01	
p^B	< 0.02	< 0.01	

Note: Results are expressed as μmoles/min/g wet wt. (mean ± SEM).

p=comparison of the means at different ethanol concentration. p^A, p^B=comparison of the respective means of (A) and (B) of the two groups at the same ethanol concentration.

Factors Contributing to the Adaptive Increase in Ethanol Oxidation After Chronic Consumption of Ethanol*

Arthur I. Cederbaum

The Alcohol Dehydrogenase Pathway of Ethanol Oxidation

The major portion of ethanol metabolism is attributed to alcohol dehydrogenase, an enzyme of the cytosol. Oxidation of ethanol by alcohol dehydrogenase generates NADH in the cytoplasm; reoxidation of NADH may be rate limiting for the overall metabolism of ethanol (Theorell and Chance 1951; Vitale et al. 1954). Compounds that oxidize NADH, such as pyruvate, D-glyceraldehyde, methylene blue, and fructose, increase the rate of ethanol metabolism. Normally, cytoplasmic processes appear insufficient to reoxidize all the NADH generated by ethanol metabolism; consequently, mitochondrial oxidation of the reducing equivalents of NADH is required. Because intact mitochondria are impermeable to NADH (Lehninger et al. 1960), reducing equivalents are transported into the mitochondria via substrate shuttles. Three major shuttle mechanisms for the transport of reducing equivalents into the mitochondria have been proposed. These are (a) the α-glycerophosphate shuttle, (b) the malate-aspartate shuttle, and (c) the fatty acid elongation shuttle (Borst 1963; Bucher and Klingenberg 1958; Klingenberg and Bucher 1961; Sactor and Dick 1960; Whereat et al. 1969). In general, the shuttles are regulated by the activities of the cytosolic and mitochondrial enzymes that comprise the shuttle, for example, α-glycerophosphate dehydrogenase, malate dehydrogenase, aspartate aminotransferase, and by the ability of the oxidized or reduced metabolites to transverse the mitochondrial membrane. All three shuttles have been reconstituted in

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NOTE: Figures and tables appear at end of paper.

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in vitro systems, using isolated liver mitochondria and the extramitochondrial components of the shuttles (Cederbaum, Lieber, Beattie, and Rubin 1973; Chappell 1968; Dionisi et al. 1970; Grunnet 1970; Haslam and Krebs 1968; Hassinen 1967; Williamson et al. 1971).

In view of the above considerations, the activity of the alcohol dehydrogenase pathway of ethanol metabolism can be affected by (a) the activity of alcohol dehydrogenase; (b) the activity of substrate shuttle systems transporting reducing equivalents into the mitochondria; and (c) the ability of the mitochondrial respiratory chain to reoxidize the reducing equivalents.

Regulation of the Alcohol Dehydrogenase Pathway in Isolated Hepatocytes

In studies with isolated systems such as liver slices (Videla and Israel 1970), perfused livers (Papenberg et al. 1970), or hepatocytes (Cederbaum et al. 1977; Meijer et al. 1975; Williamson et al. 1974), the rate of ethanol metabolism was shown to be limited by the transfer and reoxidation of reducing equivalents by the mitochondrial respiratory chain rather than by the activity of alcohol dehydrogenase. For example, in isolated hepatocytes from male Sprague-Dawley rats, pyruvate (acting as an NADH-oxidizing agent) increased the rate of ethanol metabolism by 153 percent (Cederbaum et al. 1977), suggesting that cofactor availability, that is, the reoxidation of NADH to NAD^+, and not the activity of alcohol dehydrogenase, was rate limiting for ethanol oxidation in these preparations. However, it is not clear whether the rate-limiting step in ethanol oxidation reflects the transfer of reducing equivalents into the mitochondria as catalyzed by substrate shuttles or the ability of the respiratory chain to reoxidize the reducing equivalents. The concept that the concentration of substrates that participate in the malate-aspartate shuttle play an important role in ethanol metabolism has been suggested by Williamson and collaborators (Meijer et al. 1975; Williamson et al. 1974). In the fasted state, in which the content of shuttle substrates is presumably low, ethanol oxidation may be limited by the activity of the shuttles. In the fed state, in which the content of shuttle substrates is presumably sufficient, ethanol oxidation may be limited by the capacity of the mitochondrial respiratory chain (Meijer et al. 1975; Williamson et al. 1974). A series of experiments carried out in our laboratory to evaluate those factors that are limiting for ethanol oxidation in hepatocytes prepared from fed and 24-hour starved rats is shown in table 1. The rate of ethanol oxidation was about 40 percent greater in hepatocytes from fed rats compared to hepatocytes from fasted rats. If oxygen consumption, that is, the ability of the respiratory chain to reoxidize reducing equivalents, is rate limiting for ethanol oxidation, the addition of uncoupling agents, which stimulate oxygen uptake, should increase the rate of ethanol oxidation. FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone) is a potent uncoupling agent; oxygen consumption by the liver cells was increased by 30 to 50 percent in the presence of FCCP. In liver cells prepared from fed rats, 1 and 2 μM FCCP stimulated the rate of ethanol oxidation (table 1). The stimulatory effect was lost at higher concentrations of FCCP, and inhibition was observed at 5 μM FCCP. Careful titration is therefore required to observe the stimulatory effect of FCCP. By contrast, in liver cells prepared from fasted rats, ethanol oxidation was inhibited by concentrations of FCCP, which were stimulatory with liver cells prepared from fed rats (table 1, experiment A).

The addition of the components of the malate-aspartate shuttle or the α-glycerophosphate shuttle to cells from fasted rats increased the rate of ethanol oxidation by 30 to 60 percent (table 1, experiment B). In hepatocytes from fed rats, the addition of the components of the malate-aspartate shuttle caused a lesser extent of stimulation of ethanol oxidation (25 to 33 percent) than found with liver cells from fasted rats; α-glycerophosphate was not stimulatory with preparations from fed rats (table 1, experiment B).

These results suggest that under conditions of sufficient intracellular levels of intermediates of the substrate shuttles, ethanol oxidation is regulated by the capacity of the respiratory chain to reoxidize reducing equivalents generated by the alcohol dehydrogenase reaction. This can be further seen from the results of experiment C (table 1). In hepatocytes from fed rats, the addition of malate or glutamate increased the rate of ethanol oxidation by 31 and 33 percent, respectively. However, in the presence of malate or glutamate plus the uncoupling agent dinitrophenol, the rate of ethanol oxidation was increased by 60 percent and 105 percent, respectively. In hepatocytes from fasted rats, the addition of malate or glutamate increased the rate of ethanol oxidation by 58 percent and 59 percent, respectively. In the presence of malate or glutamate plus dinitrophenol, the rate of ethanol oxidation was increased by 93 percent and 136 percent, respectively. Consequently, in the presence of substrate shuttle metabolites, uncoupling agents are effective in stimulating the rate of ethanol oxidation beyond the stimulation produced by the substrate shuttle components themselves. In summary, the concentration of substrate shuttle intermediates is rate limiting for the oxidation of ethanol by liver cells from fasted rats, whereas in liver cells from fed rats, the rate of ethanol oxidation is regulated by mitochondrial reoxidation of reducing equivalents.

It is important to note that none of the above conditions (pyruvate, shuttle metabolites, uncoupling agents, or metabolites plus uncouplers) increased the rate of ethanol oxidation to a higher rate than the reported in vivo rate (about 200 μmol/h/g, wet wt.). Consequently, under certain conditions (maximal metabolite levels and oxygen consumption rates), conditions more associated with in vivo than in vitro conditions, ethanol oxidation may also be limited by the maximum capacity of alcohol dehydrogenase (Crow et al. 1977; Plapp 1975).

Ethanol Metabolism After Chronic Ethanol Consumption

Chronic administration of ethanol increases the rate of ethanol metabolism (Bernstein et al. 1973; Hawkins et al. 1966; Lieber and DeCarli 1970, 1972; Tobon and Mezey 1971). The mechanisms underlying this increase are controversial. With regard to the effects of chronic ethanol consumption on the activity of enzyme systems that oxidize ethanol to acetaldehyde, alcohol dehydrogenase activity has been reported to be increased (Hawkins et al. 1966; Mistilis and Birchall 1969), not changed (Videla and Israel 1970), or even slightly decreased (Lieber and DeCarli 1968), after chronic ethanol intake. The activity of the microsomal ethanol oxidizing system was increased after chronic ethanol consumption (Lieber and DeCarli 1970, 1972). It has been suggested that MEOS can account for about two-thirds of the adaptive increase in ethanol oxidation found after chronic ethanol administration (Lieber and DeCarli 1970, 1972).

In view of altered mitochondrial structure as evidenced by ultrastructural examination (Rubin et al. 1970) and reports of increased membrane permeability (French 1968), it has been suggested that the normal permeability barrier to NADH had been altered by the ethanol treatment and that transport of substrate metabolites into and out of the mitochondria may be augmented (Rawat and Kuriyama 1972). Therefore, enhanced NADH permeability and shuttle activities would contribute to the enhanced rate of ethanol metabolism. Since about 60 percent of the oxygen consumption in the liver is utilized to reoxidize NADH formed from the oxidation of ethanol (Lindros et al. 1972), increased mitochondrial activity or content per gram of tissue may play a role in the adaptive increase in ethanol metabolism. In this regard, a major hypothesis to account for the metabolic increase in ethanol metabolism was advanced by Israel and collaborators (Bernstein et al. 1973, 1974; Israel, Videla, and Bernstein 1975). Chronic ethanol consumption was suggested to result in the production of a hypermetabolic state of the liver, thereby accelerating the reoxidation of NADH produced by the alcohol dehydrogenase pathway. The hypermetabolic state was hypothesized to result from an increase in ($Na^+ + K^+$)-activated ATPase, the increase in generation of ADP bringing the hepatocyte closer to state 3, thereby increasing mitochondrial oxygen consumption. This ethanol-induced hypermetabolic state may be mediated by thyroid hormones and possibly by catecholamines. In fact, the antithyroid drug propylthiouracil has been used to prevent liver cell damage produced by ethanol (Israel, Kalant, Orrego, Khanna, Videla, and Phillips 1975).

To evaluate some of the above possibilities (Cederbaum, Lieber, Toth, Beattie, and Rubin 1973; Cederbaum et al. 1974; Cederbaum, Lieber, Beattie, and Rubin 1975), mitochondria were isolated from male Sprague-Dawley rats that were fed a liquid diet for about 4 weeks and in which ethanol provided 36 percent of total calories; protein, 16 percent; fat, 35 percent; and carbohydrates, 13 percent. Pair-fed littermates were given a similar diet except that carbohydrates replaced ethanol isocalorically (DeCarli and Lieber 1967). This diet has been shown to result in the production of a fatty liver (DeCarli and Lieber 1967). As shown in table 2, the rate of blood ethanol clearance was increased by 44 percent in rats fed ethanol chronically. However, the activity of alcohol dehydrogenase was decreased 20 percent, indicating the increased rate of ethanol metabolism is not mediated by a parallel increase in the activity of this enzyme. The activity of enzymes participating in the malate-aspartate or α-glycerophosphate shuttles were either not affected or were slightly decreased by chronic ethanol feeding (table 2). When the shuttles were reconstituted with mitochondria from ethanol-fed rats or from pair-fed controls, identical rates of NADH oxidation were observed (table 2).

Further studies on mitochondrial permeability indicated that various inhibitors of anion transport across the mitochondrial membrane, e.g., iodobenzylmalonate, avenaciolide, and mersalyl, were effective in preventing malate-aspartate shuttle activity to comparable extents with mitochondria from ethanol-fed rats and controls (Cederbaum, Lieber, Toth, Beattie, and Rubin 1973). Moreover, swelling studies in isotonic solutions of ammonium salts (an assay of the penetration of anions into the mitochondria, which are freely permeable to NH_3) indicated that mitochondria from control rats swelled to the same extent as those ethanol-fed rats when suspended in the ammonium salts of phosphate, malate, succinate, glutamate, or citrate

(Cederbaum, Lieber, Toth, Beattie, and Rubin 1973). Thus, chronic ethanol consumption produced no change in the permeability of the mitochondria to these anions. In addition, the normally low permeability of intact mitochondria to NADH was maintained by mitochondria from ethanol-fed rats (table 2).

To evaluate the possible role of mitochondrial function in the increased rate of ethanol metabolism, studies of energy production and utilization by isolated mitochondria were carried out (Cederbaum et al. 1974, 1976; Cederbaum, Lieber, Beattie, and Rubin 1975). The ability of chronic ethanol consumption to cause proliferation of mitochondria, as is known to occur with the endoplasmic reticulum (Iseri et al. 1966), was carried out to assess "total mitochondrial activity."

The amount of mitochondrial protein per gram of liver can be calculated from the total activity of an enzyme in the homogenate (units/gram liver) and the specific activity of the enzyme in the mitochondria (units/milligram mitochondrial protein). The effects of chronic ethanol feeding on the activities of cytochrome oxidase and succinic dehydrogenase, enzymes found only in the mitochondria, were studied. Total and specific activities of cytochrome oxidase and succinic dehydrogenase in the mitochondria and in homogenates were reduced by ethanol feeding (figures 1 and 2). Total hepatic protein, mitochondrial protein per gram of liver, and the yield of mitochondria were not affected by ethanol feeding, as calculated from the activities of succinic dehydrogenase and cytochrome oxidase (figures 1 and 2). Thus, chronic ethanol feeding does not increase the content of mitochondrial protein nor the yield of mitochondria. The effect of chronic ethanol consumption on the oxidation of (a) NAD^+-dependent substrates (glutamate, α-ketoglutarate); (b) succinate, which is flavin-linked; and (c) ascorbate, which reduces cytochrome *c*, was studied. Mitochondrial oxygen consumption (assayed polarographically) was measured in the resting state (state 4) and the ADP-energized state (state 3).

Under state 4 conditions, there was a slight decrease in the oxidation of all substrates (Cederbaum et al. 1974; Cederbaum, Lieber, Beattie, and Rubin 1975). This decrease may be due, at least in part, to the decrease in the activities of cytochrome oxidase or succinic dehydrogenase (figures 1 and 2) or the previously demonstrated decrease in the mitochondrial contents of cytochromes *b*, *a*, and a_3 (Rubin et al. 1970). The addition of ADP increased the rate of oxidation of all the substrates. However, mitochondria from ethanol-fed rats again showed a lesser rate of oxygen consumption than did mitochondria from controls (figure 3). Respiratory control and P/O ratios were depressed with the NAD^+-dependent substrates (Cederbaum et al. 1974). Other energy-dependent functions were also depressed after chronic ethanol consumption, e.g., energized Ca^{2+} uptake (Cederbaum et al. 1974) and reversed electron transport (Cederbaum et al. 1976). Moreover, the oxidation of other substrates, e.g., fatty acids (Cederbaum, Lieber, Beattie, and Rubin 1975) and citric acid cycle intermediates (Cederbaum et al. 1976) was also depressed after chronic ethanol consumption. These results indicate that chronic ethanol consumption decreases, rather than increases, isolated mitochondrial activity.

In summary, the increased rate of ethanol metabolism found after chronic ethanol consumption does not correlate with the activity of alcohol dehydrogenase, in vitro mitochondrial activity, content of mitochondria, enhanced shuttle activity, or permeability to various substrate shuttle anions and NADH.

Since various results obtained with isolated mitochondria did not correlate with the increased rate of ethanol oxidation, studies with isolated liver cells prepared from the ethanol-treated rats were undertaken (Cederbaum et al. 1978). The rate of ethanol oxidation in liver cells isolated from ethanol-fed rats was 78 μmol/h/g liver, wet wt., compared to a value of 56 μmol/h/g liver for controls (table 3). The increase of 39 percent in the rate of ethanol oxidation is comparable to that found in vivo (table 2), indicating that the increase in ethanol metabolism involves pathways associated with the liver. Methylpyrazole, a potent inhibitor of alcohol dehydrogenase, decreased the rate of ethanol metabolism by 86 percent in liver cells isolated from controls and by 75 percent in liver cells from ethanol-fed rats (table 3). These results confirm the dominant role of the alcohol dehydrogenase pathway in both liver cell preparations. However, the results also suggest that a nonalcohol dehydrogenase-dependent pathway may contribute a larger fraction to the overall pathway of ethanol metabolism in the liver cells from the ethanol-fed rats. Indeed, about one-half of the increase in the rate of ethanol metabolism persisted in the presence of concentrations of methylpyrazole that totally block the activity of alcohol dehydrogenase. Similar results were obtained with other inhibitors of the alcohol dehydrogenase pathway, that is, about 40 to 50 percent of the increase in the rate of ethanol oxidation was not abolished by inhibitors of the respiratory chain (rotenone, antimycin), inhibitors of ADP-dependent oxygen consumption (oligomycin, atractyloside), and inhibitors of substrate shuttle anion transport (amino-oxyacetate, avenaciolide, iodobenzylmalonate) (table 3). Furthermore, the entire increase in the rate of ethanol oxidation persisted in the presence of various substrate shuttle metabolites, in the absence or presence of an uncoupling agent (dinitrophenol), suggesting that the increased rate did not reflect differences in hepatic content of these metabolites (table 3).

There was no effect by ouabain, an inhibitor of the ($Na^+ + K^+$)-activated ATPase, on the increased rate of ethanol oxidation (table 3), suggesting that increased activity of this enzyme, with the subsequent development of a state 3 condition, did not occur. Indeed, hepatic oxygen consumption with a variety of substrates, or in the presence of dinitrophenol, was slightly decreased, not increased, after chronic ethanol consumption (table 4). These results are, in general, not consistent with the development of a hypermetabolic state after chronic ethanol consumption. In view of the demonstrated increase in MEOS activity after chronic ethanol consumption (Lieber and DeCarli 1970, 1972), the greater extent of increase in ethanol metabolism at higher ethanol concentrations (Matsuzaki and Lieber 1976), the inability of pyrazole and methylpyrazole to suppress the increased rate of ethanol metabolism (Cederbaum et al. 1978; Lieber and DeCarli 1972; Matsuzaki and Lieber 1976), and the persistence of at least part of the increased rate of ethanol metabolism in the presence of various inhibitors of the alcohol dehydrogenase pathway (table 3), alcohol dehydrogenase-independent pathways appear to contribute to the metabolic adaptation that occurs after chronic ethanol consumption.

Summary

These results demonstrate that the rate of ethanol oxidation may be regulated by different factors, depending on the reaction conditions. Therefore, in evaluating pathways and mechanisms that play a role in the increased rate of ethanol oxidation found after chronic ethanol consumption, all these various factors should be measured. Under conditions used in this study, the increased rate of ethanol oxidation was not associated with an increased activity of alcohol dehydrogenase, with increased mitochondrial content or activity, with enhanced permeability to NADH and substrate shuttle anions, or with increased shuttle activity. Part of the increased rate of ethanol oxidation persisted in the presence of various inhibitors of the alcohol dehydrogenase pathway, including methylpyrazole. It is possible that the metabolic adaptation may involve alcohol dehydrogenase-dependent and -independent pathways. Transhydrogenation between $NADP^+$ and NADH (e.g., the malic enzyme reaction) may link the two pathways. Further studies are required to determine if these "linking" pathways contribute to the metabolic adaptation. No evidence for a hypermetabolic state was found under our reaction conditions. Therefore, chronic ethanol consumption can lead to an accelerated rate of ethanol metabolism without the establishment of a hypermetabolic state.

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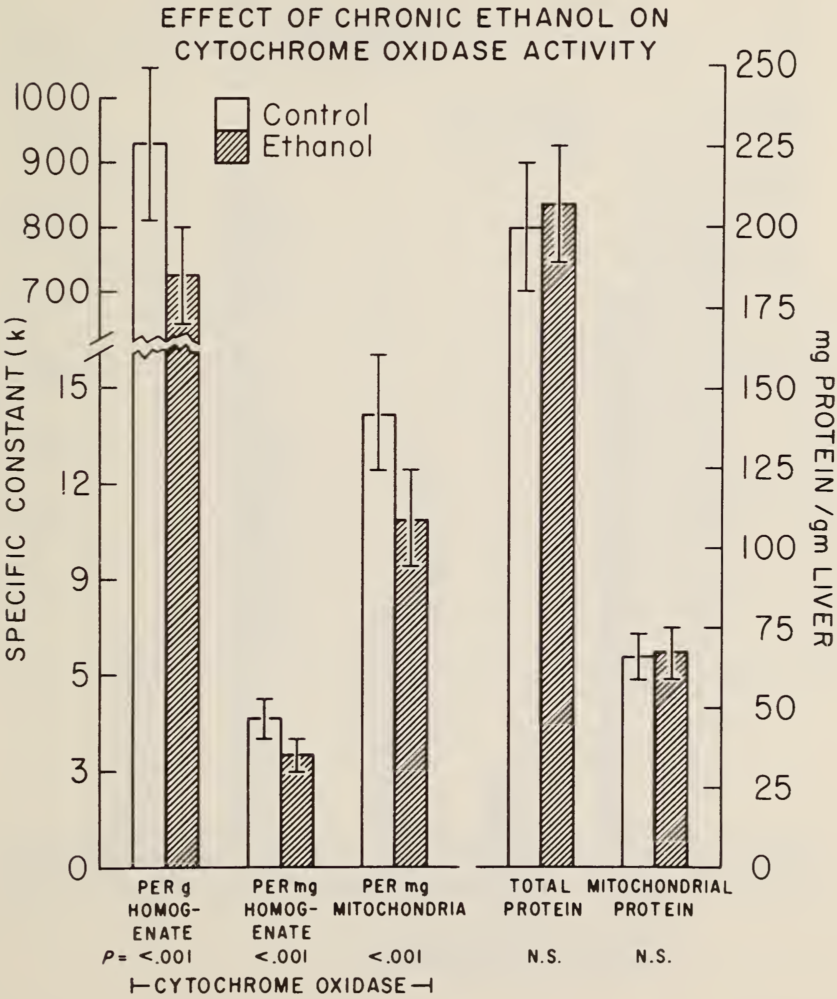
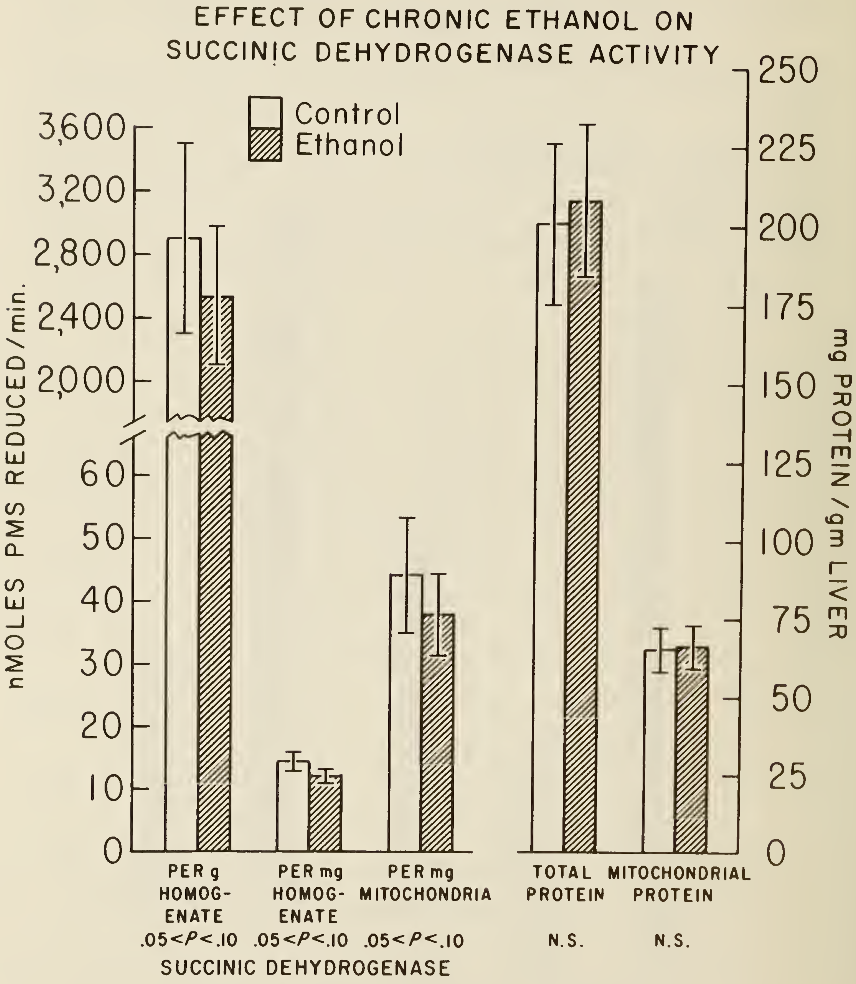
Figure 1. Effect of Chronic Ethanol Consumption on the Activity of Cytochrome Oxidase and on the Yield of Mitochondrial Protein



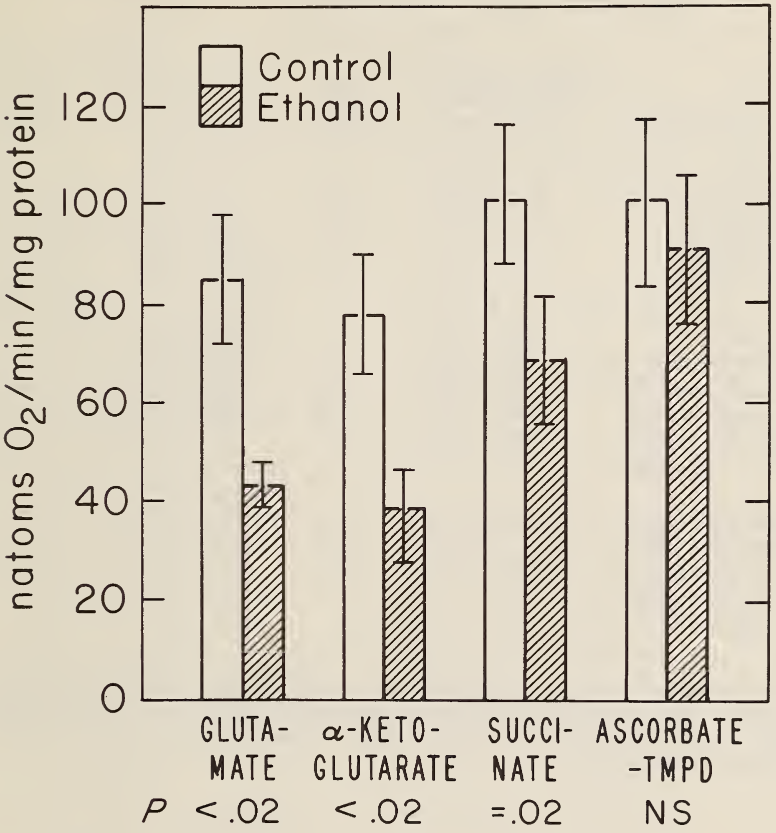
(From Cederbaum, A.I.; Lieber, C.S.; Toth, A.; Beattie, D.S.; and Rubin, E. Effects of ethanol and fat on the transport of reducing equivalents into mitochondria. *J Biol Chem*, 248:4977-4986, 1973. Copyright 1973 by the American Society of Biological Chemists, [Beth., MD].)

Figure 2. **Effect of Chronic Ethanol Consumption on the Activity of Succinic Dehydrogenase and on the Yield of Mitochondrial Protein**



(From Cederbaum, A.I.; Lieber, C.S.; Toth, A.; Beattie, D.S.; and Rubin, E. Effects of ethanol and fat on the transport of reducing equivalents into mitochondria. *J Biol Chem*, 248:4977-4986, 1973. Copyright 1973 by the American Society of Biological Chemists, [Beth., MD].)

Figure 3. Effect of Chronic Ethanol Consumption on the Oxidation of Various Substrates by Isolated Liver Mitochondria



Results refer to state 3 (presence of ADP) respiration. (From Cederbaum, A.I.; Lieber, C.S.; and Rubin, E. Effect of chronic ethanol treatment on mitochondrial functions. *Arch Biochem Biophys*, 165:560-569, 1974. Copyright 1974 by Academic Press, Inc. [NY].)

Table 1. Effect of Components of Substrate Shuttles and Uncoupling Agents on Ethanol Oxidation by Hepatocytes Isolated From Fed and Fasted Rats

	Fed		Fasted	
Addition	Ethanol Oxidation (μmol/h/g wet wt)	Effect (percent)	Ethanol Oxidation (μmol/h/g wet wt)	Effect (percent)
A. None	68.5 ± 10.7	--	47.4 ± 3.1	--
1 μM FCCP	91.1 ± 10.8	+ 33	--	--
2 μM FCCP	91.1 ± 8.2	+ 33	39.5 ± 6.1	− 17
3 μM FCCP	74.1 ± 9.6	+ 8	31.3 ± 5.0	− 34
5 μM FCCP	39.3 ± 5.7	− 43	23.4 ± 2.1	− 51
B. None	57.00 ± 4.82	--	41.25 ± 2.23	--
10 mM malate	74.57 ± 4.36	+ 31	65.25 ± 5.40	+ 58
10 mM aspartate	71.00 ± 5.78	+ 25	60.16 ± 6.16	+ 46
10 mM glutamate	75.75 ± 4.68	+ 33	65.50 ± 3.72	+ 59
10 mM α-glycerophosphate	51.28 ± 5.33	− 10	52.00 ± 6.56	+ 26
C. 0.05 mM Dinitrophenol + malate	91.00 ± 7.13	+ 60	79.66 ± 6.11	+ 93
0.05 mM Dinitrophenol + glutamate	116.60 ± 10.44	+105	97.33 ± 9.78	+136

(From Cederbaum, A.I.; Dicker, E.; and Rubin, E. Transfer and reoxidation of reducing equivalents as the rate-limiting steps in the oxidation of ethanol by liver cells isolated from fed and fasted rats. *Arch Biochem Biophys,* 183:638-646, 1977. Copyright 1977 by Academic Press, Inc. [NY].)

Table 2. Effect of Chronic Ethanol Consumption on Ethanol Metabolism, Alcohol Dehydrogenase, Mitochondrial Activity, Shuttle Enzymes, and Reconstituted Shuttle Activities

Reaction	Specific Activity		Effect of Ethanol (percent)
	Control	Ethanol Treated	
Blood ethanol clearance (μmol/min/kg body wt.)	90 ± 9	130 ± 17	+44
Alcohol dehydrogenase (nmol/(min/mg soluble protein)	13.8 ± 1.5	11 ± 1	−20
Mitochondrial aspartate aminotransferase (nmol/min/mg mitochondrial protein)	1141 ± 66	1156 ± 10.6	+ 1
Cytosolic aspartate aminotransferase (nmol/min/mg soluble protein)	114 ± 6	118 ± 4	+ 4
Mitochondrial α-glycerophosphate oxidase (natom/min/mg mitochondrial protein)	9.8 ± 1.2	7.4 ± 1	−25
Cystolic α-glycerophosphate dehydrogenase (nmol/min/mg soluble protein)	190 ± 20	150 ± 20	−21
NADH oxidation (nmol/min/mg mito protein)	3.91 ± 0.63	3.60 ± 0.58	− 8
Reconstituted fatty acid shuttle (nmol/min/mg mito protein)	11.2 ± 1.3	11.1 ± 1.4	− 1
Reconstituted α-glycerophosphate shuttle (nmol/min/mg mito protein)	16 ± 3.2	14.1 ± 1.4	−12
Reconstituted malate-aspartate shuttle (nmol/min/mg mito protein)	17.1 ± 1.6	18.5 ± 2.1	+ 8

(From Cederbaum, A.I.; Lieber, C.S.; Toth, A.; Beattie, D.S.;and Rubin, E. Effects of ethanol and fat on the transport of reducing equivalents into mitochondria. *J Biol Chem*, 248:4977-4986, 1973. Copyright 1973 by American Society of Biological Chemists [Beth., MD].)

Table 3. Rate of Ethanol Metabolism in Liver Cells Isolated From Chronic Ethanol-Fed Rats and Pair-Fed Controls

Addition	Rate of Ethanol Oxidation: Control (μmol/h/g liver, wet wt.)	Rate of Ethanol Oxidation: Chronic-Ethanol (μmol/h/g liver, wet wt.)	Increase in Ethanol Oxidation
A. None	56.5 ± 3.6	78.4 ± 3.9	21.9
Methylpyrazole	8.0 ± 2.3	17.5 ± 1.5	9.5
Rotenone	32.7 ± 3.8	44.0 ± 14.3	11.3
Antimycin	14.4 ± 3.5	23.8 ± 2.3	9.4
Oligomycin	30.5 ± 3.6	39.5 ± 2.8	9.4
Atractyloside	33.0 ± 4.8	45.6 ± 4.1	12.6
Amino-oxyacetate	32.5 ± 4.3	43.9 ± 3.9	11.4
Avenaciolide	32.8 ± 3.9	44.8 ± 3.4	12.0
Iodobenzylmalonate	26.2 ± 5.6	36.5 ± 3.1	10.3
Ouabain	42.5 ± 4.6	62.9 ± 5.5	20.4
B. None	54.0 ± 3.7	69.6 ± 5.4	15.6
Malate	60.1 ± 5.7	80.2 ± 9.8	20.1
Aspartate	60.3 ± 3.6	83.3 ± 6.4	23.0
Glutamate	60.0 ± 3.9	74.3 ± 5.6	14.3
α-Glycerophosphate	57.7 ± 4.3	71.1 ± 5.3	13.4
Aspartate + Dinitrophenol	72.9 ± 3.6	91.1 ± 5.2	18.2
Glutamate + Dinitrophenol	103.6 ± 5.8	124.0 ± 9.1	20.3
α-Glycerophosphate + Dinitrophenol	71.2 ± 6.2	88.7 ± 7.9	17.5

(From Cederbaum, A.I.; Dicker, E.; Lieber, C.S.; and Rubin, E. Ethanol oxidation by isolated hepatocytes from ethanol-treated and control rats; factors contributing to the metabolic adaptation after chronic ethanol consumption. *Biochem Pharmacol*, 27:7-15, 1978. Copyright 1978 by Pergamon Press, Inc. [Elmsford, NY].)

Table 4. Rate of Oxygen Consumption in Liver Cells Isolated From Chronic Ethanol-Fed Rats and Pair-Fed Controls

Addition	Rate of Oxygen Consumption	
	Control	Chronic Ethanol
	(μmol/min/g liver, wet wt.)	
None	2.45 ± 0.13	2.22 ± 0.21
Glutamate	2.79 ± 0.17	2.53 ± 0.23
Pyruvate	2.49 ± 0.09	2.19 ± 0.41
α-Glycerophosphate	2.88 ± 0.18	2.66 ± 0.22
Succinate	4.35 ± 0.37	4.02 ± 0.44
Dinitrophenol	3.36 ± 0.28	3.01 ± 0.39

(From Cederbaum, A.I.; Dicker, E.; Lieber, C.S.; and Rubin, E. Ethanol oxidation by isolated hepatocytes from ethanol-treated and control rats; factors contributing to the metabolic adaptation after chronic ethanol consumption. *Biochem Pharmacol*, 27:7-15, 1978. Copyright 1978 by Pergamon Press, Inc. [Elmsford, NY].)

Some Observations on Ethanol Metabolism in Alcohol Dehydrogenase Negative Deermice

Michael R. Felder and Karen G. Burnett

Introduction

The liver is the primary site of ethanol oxidation. Studies using pyrazole, an alcohol dehydrogenase (ADH) inhibitor, suggest that virtually all ethanol oxidation at low concentrations is mediated through this ADH pathway (Grunnet et al. 1973). At higher concentrations, considerable ethanol oxidation occurs in the presence of pyrazole, suggesting alternate routes for ethanol elimination (Lieber and DeCarli 1970, 1972; Rognstad 1974). Numerous reports provide strong evidence that the microsomal fraction of the hepatocyte can carry out ethanol oxidation at higher concentrations of ethanol. This microsomal ethanol oxidizing system (MEOS) has been extensively characterized (Lieber and DeCarli 1970, 1974; Miwa et al. 1978; Ohnishi and Lieber 1977; Teschke et al. 1974, 1975*a*,*b*, 1976).

This MEOS activity has been reconstituted from partially purified cytochrome P-450, NADPH-cytochrome c reductase, and L-∝-dioleoyl lecithin (Miwa et al. 1978; Ohnishi and Lieber 1977). Chronic ethanol feeding of rats induces qualitative and quantitative alterations in the cytochrome P-450 compared to pair-fed controls (Ohnishi and Lieber 1977). Rates of ethanol clearance from blood and MEOS activity are both known to increase with ethanol consumption (Lieber and DeCarli 1970). Other workers, however, have argued that MEOS activity is due entirely to catalase contamination of isolated microsomes and in the reconstituted system. Using aminotriazole-treated rats to inhibit catalase and methylpyrazole to inhibit ADH, Thurman et al. (1975) demonstrated a complete lack of ethanol oxidation in the perfused liver from such treated animals. However, either of these drugs could conceivably have an effect on the MEOS system, as suggested by these authors. The fact that acatalasemic mice have MEOS activity (after mild heat treatment to totally inactivate the labile catalase in the microsomes) strongly supports the contention that MEOS is a separate and distinct pathway from catalase (Lieber and DeCarli 1974; Teschke et al. 1975*b*).

NOTE: Figures and tables appear at end of paper.

A potentially useful mammalian model that circumvents the use of ADH inhibitors is the ADH-negative *Peromyscus maniculatus* (deermouse) stocks developed in this laboratory (Burnett and Felder 1978*a*,*b*; Felder 1975). ADH-negative and ADH-positive animals were found in a laboratory-reared population of deermice maintained by Dr. Wallace Dawson of this department. True breeding stocks of the two phenotypes have been obtained by breeding and progeny testing.

This brief review will provide a summary of the genetics of ADH in these mice. In addition, a summary of the preliminary work on ethanol metabolism in ADH-negative and ADH-positive animals will be presented.

Genetic Control of Liver Alcohol Dehydrogenase in the Deermouse

Two ADH phenotypes have been observed in the *P. maniculatus* laboratory population: ADH-negative and ADH-positive animals. *P. polionotus* liver ADH is electrophoretically distinct ("slow" versus "fast" phenotypes) from the *P. maniculatus* enzyme. These three electrophoretic phenotypes are shown in figure 1. ADH-negative animals also contain no detectable ADH activity when measured spectrophotometrically (Burnett and Felder 1978*a*). The subbanding of liver ADH is virtually eliminated by preincubation with NADH (figure 2). The ADH phenotypes of the parental stocks and F_1 hybrids are also illustrated in figure 2.

Several points about these phenotypes may be made. Progeny of ADH-"slow" × ADH-"fast" crosses exhibit a three-banded phenotype. This is expected if ADH is a dimeric enzyme. ADH-"slow" and ADH-"fast" parents crossed to ADH-negative animals produce only "slow" and "fast" phenotypic offspring, respectively. Such observations suggest that ADH-negative animals are not producing an inactive polypeptide capable of dimerizing with either "slow" or "fast" polypeptides to produce an active, distinct molecular species.

P. maniculatus and *P. polionotus* are easily crossed and produce fertile offspring. Extensive genetic analysis (Burnett and Felder 1978*a*) has determined the genetic nature of these phenotypes. The *Adh* gene appears to control the structure of ADH. Three alleles have been detected—Adh^S, Adh^F, and Adh^N—and these encode slow migrating, fast migrating, and negative (or null) ADH activity, respectively. Whether or not Adh^N represents a regulatory site closely linked to the structural gene for ADH cannot be currently answered. Well over 100 offspring of Adh^N/Adh^S × Adh^S/Adh^S crosses have been examined without evidence of a recombination event that restores the activity of a "fast" polypeptide. This might be expected to occur only rarely, however, if the two sites are closely linked.

The "fast" and "slow" forms of ADH have been purified to homogeneity, and antisera produced in rabbits to each purified enzyme have been used to search for the presence of antigenically cross-reacting material in the liver of ADH-negative animals (Burnett and Felder 1978*b*). These antisera do not precipitate radiolabeled protein from ADH-negative animals, nor do liver extracts from ADH-negative mice alter the equivalence points of these antisera when titrated with ADH activity from "slow" or "fast" animals (Burnett and Felder 1978*b*). In addition, precipitin lines fail to form when either antiserum is tested with ADH-negative liver supernatants by the Ochterlony double diffusion method (figure 3).

Ethanol Metabolism in ADH-Negative and ADH-Positive Deermice

This animal model allows ethanol oxidation to be studied in the presence and absence of liver ADH activity without using inhibitors. As an initial means of measuring the extent of ethanol metabolism in the two genotypes of animals, ethanol-induced sleep times were measured. Negative animals (genotype Adh^{N}/Adh^{N}) sleep nearly twice as long (138.2 min) as do ADH-positive animals (genotype Adh^{F}/Adh^{F}; sleeptime 76.1 min) when given a weight-specific hynotic dose of ethanol (Burnett and Felder 1980).

Differences in sleeptime between the two groups of animals are consistent with the differences in ethanol oxidation rates between the two genotypes of animals as measured by the blood ethanol elimination rate (BEER). In addition, the effects of a 10 percent ethanol solution as sole source of liquid for 6 weeks in both genotypes of mice were also investigated (table 1). These results (Burnett and Felder 1980) confirm that animals lacking ADH can still oxidize substantial quantities of ethanol at high blood ethanol levels; this rate is about two-thirds the rate found in ADH-positive animals. Ethanol feeding increases BEER in ADH-positive animals ($p<0.005$) but does not in ADH-negative mice.

The MEOS activity of both genotypes under control and ethanol-fed conditions was also examined (Burnett and Felder 1980). Catalase activity was measured because it has been implicated in ethanol metabolism. The data in table 2 show that MEOS is more than twofold higher in Adh^{N}/Adh^{N} than in Adh^{F}/Adh^{F} animals under control conditions.

This is true whether the activity is expressed on a wet weight or milligram microsomal protein basis. Ethanol feeding increases MEOS activity more than threefold in both strains of animals, and the difference in MEOS activity between ethanol-fed Adh^N/Adh^N and Adh^F/Adh^F animals remains more than twofold. MEOS activity was measured with propanol as substrate in the presence of azide to minimize catalase activity, which occurs when ethanol is used as substrate (Teschke et al. 1975*a*). These data suggest that ethanol feeding increases MEOS activity and that MEOS is not due to contaminating catalase. In agreement with other studies, ethanol feeding does not increase ADH activity (Korvula and Lindros 1975; Tobon and Mezey 1971).

Several microsomal components that have been implicated in MEOS were assayed in this study (Burnett and Felder 1980). These data are shown in table 3. Ethanol-fed deermice do not have increased cytochrome P-450, NADPH cytochrome c reductase, or phospholipid, even though the MEOS is induced threefold by ethanol feeding. The cytochrome P-450 level does increase during ethanol feeding in rats to an extent that is comparable to the MEOS induction (Ishii et al. 1973). Form I of cytochrome P-450 is most strikingly increased by ethanol feeding (Hasumura et al. 1975). The ethanol feeding in rats was done using nutritionally adequate liquid diets with isocaloric substitution of ethanol, whereas the deermouse ethanol feeding was done by simply using 10 percent ethanol as the sole source of liquid. In addition, there were some differences in the means by which microsomes were isolated in these studies.

Discussion and Conclusions

The Adh^N/Adh^N genotype of the deermouse and the Adh^F/Adh^F controls provide an ideal system for studying the routes of ethanol metabolism in the absence and presence of liver alcohol dehydrogenase activity. The substantial rate of blood ethanol clearance that occurs in the absence of this enzyme activity provides a clear demonstration that alternate pathways exist for ethanol oxidation.

The MEOS activity appears to be responsible for a substantial portion of this ethanol oxidation at high ethanol concentrations. MEOS activity is increased in animals lacking alcohol dehydrogenase activity, suggesting that small amounts of ethanol produced endogenously (Krebs and Perkins 1970) when not metabolized by ADH may be capable of inducing MEOS. Ethanol feeding induces a dramatic increase in MEOS in these animals. BEER is increased only in Adh^F/Adh^F

animals, however. The increase is small but statistically significant. BEER is somewhat difficult to measure in small animals because of difficulties in obtaining blood and in the inability to simulate steady-state conditions. Taken at face value, however, these results suggest that the adaptive response to ethanol consumption resulting in increased BEER is mediated primarily through the ADH pathway. The increased activity of this pathway is thought not to be modulated by increased activity of ADH, but by increasing the reoxidation rate of NADH. Several experimental approaches have been used to increase the rate of mitochondrial oxidation, and such treatments lead to increased ethanol oxidation by liver slices (Videla and Israel 1970).

Strong evidence exists (Ohnishi and Lieber 1977) that implicates cytochrome P-450 as a component of MEOS. Our work on the deermouse does not show an increase in total cytochrome P-450 content in ethanol-fed mice even though MEOS activity is sixfold higher in ethanol-fed Adh^{N}/Adh^{N} mice than in naive Adh^{F}/Adh^{F} mice. This finding may point to a significant activation or modification of cytochrome P-450 that may occur with ethanol feeding that is not accompanied by an increase in the amount of cytochrome P-450. It will be interesting to investigate the forms of cytochrome P-450 in these two genotypes under ethanol-fed and naive conditions.

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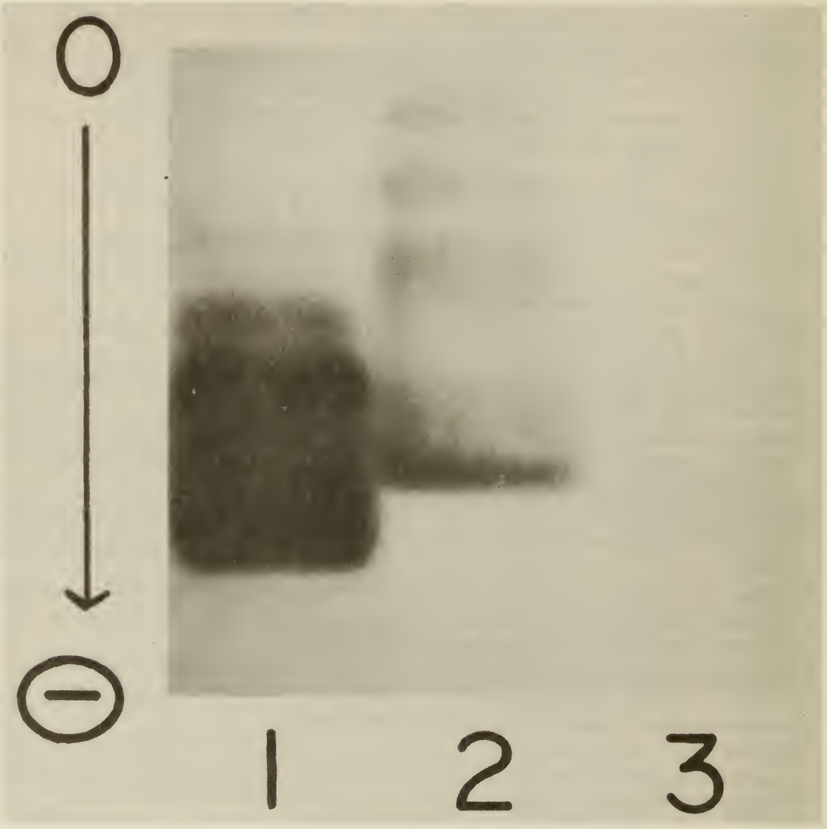
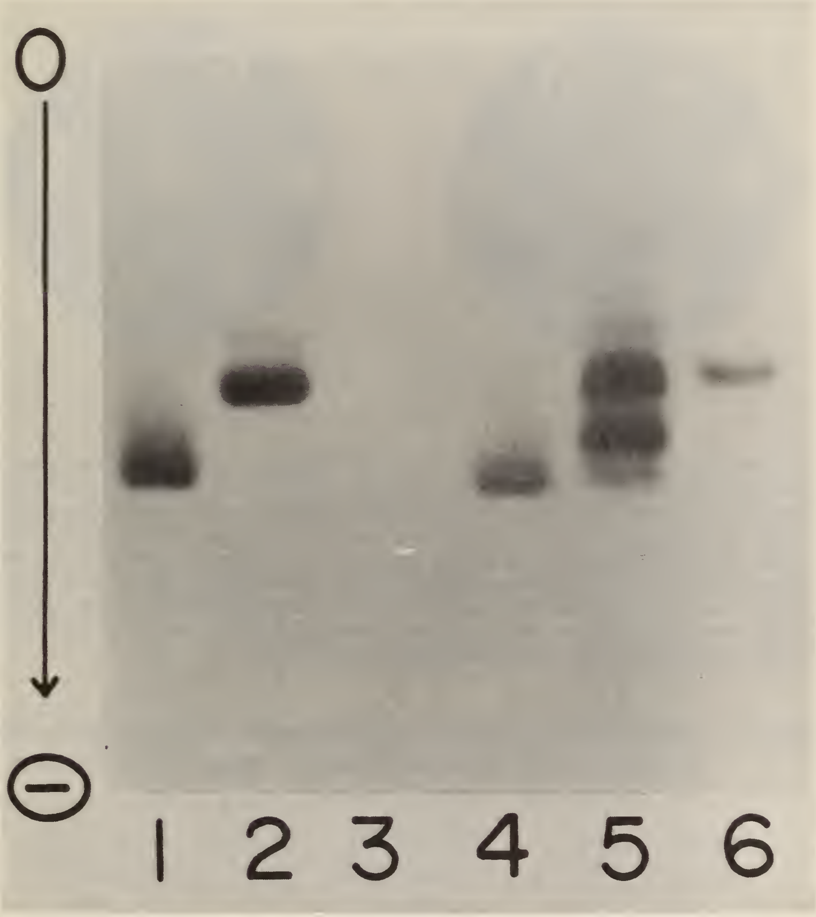
Figure 1. Liver ADH Zymogram Demonstrating Three Phenotypes in *Peromyscus* Laboratory Stocks



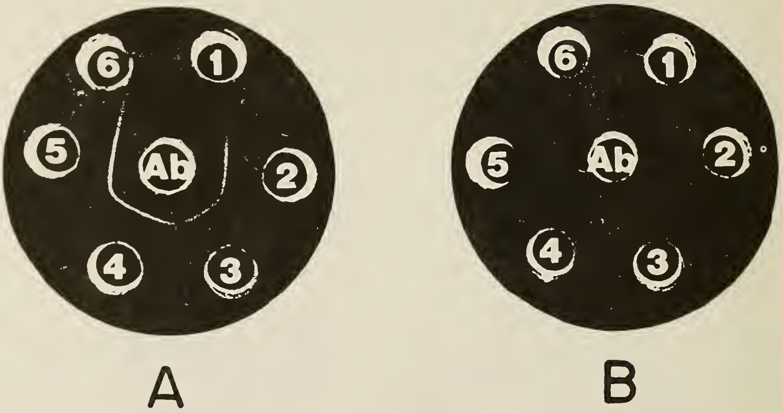
The electrophoretic phenotypes correspond to the following genotypes: (1) Adh^F/Adh^F, (2) Adh^S/Adh^S, (3) Adh^N/Adh^N. (From Burnett, K.G., and Felder, M.R. Genetic regulation of liver alcohol dehydrogenase in *Peromyscus. Biochem Genet*, 16:443-454, 1978*a*. Copyright 1978 Plenum Publishing Co. [NY].)

Figure 2. Starch Gel Zymograms of Crude *Peromyscus* Liver Homogenates



The electrophoretic phenotypes correspond to the following genotypes: (1) Adh^F/Adh^F, (2) Adh^S/Adh^S, (3) Adh^N/Adh^N, (4) Adh^F/Adh^N, (5) Adh^F/Adh^S, (6) Adh^S/Adh^N. Subbands were eliminated by addition of 1 mm NADH to liver homogenates prior to electrophoresis. (From Burnett, K.G., and Felder, M.R. Genetic regulation of liver alcohol dehydrogenase in *Peromyscus. Biochem Genet*, 16:443-454, 1978*a*. Copyright 1978 Plenum Publishing Co. [NY].)

Figure 3. Immunochemical Reactivity of Various Antigens with Anti-ADH Antiserum



Wells contain (1) liver homogenate of Adh^N/Adh^N, (2) pure fast ADH, (3) liver homogenate of Adh^S/Adh^S, (4) liver homogenate of Adh^F/Adh^F, (5) pure slow ADH, and (6) liver homogenate of Adh^N/Adh^N. The visible precipitin reaction between each antiserum and all four preparations with ADH activity demonstrates that slow and fast isozymes have similar, if not identical, immunological determinants. Antisera to the slow and fast isozymes fail to detect cross-reacting material in liver homogenates of Adh^N/Adh^N. (From Burnett, K.G., and Felder, M.R. *Peromyscus* alcohol dehydrogenase: Lack of cross-reacting material in enzyme-negative animals. *Biochem Genet,* 16:1093-1105, 1978*b*. Copyright 1978 by Plenum Publishing Co. [NY].)

Table 1. Individual Blood Ethanol Elimination Rates for Naive and Ethanol-Fed Individual of Both *P. Maniculatus* Strains*

Regimen	Adh^F/Adh^F	Adh^N/Adh^N
Naive	94.9 ± 24.9 (32)	63.9 ± 19.5 (44)
Ethanol-fed	114.3 ± 26.9 (43)	59.5 ± 18.8 (38)

*Rates are expressed as the change in blood ethanol content (mg ethanol/100 ml of blood) per hour for the time interval during which blood ethanol content remained greater than 200 mg ethanol/100 ml of blood. No significant difference was found between the blood ethanol elimination rates of male and of female *P. maniculatus* at these high blood ethanol concentrations. Values given are the average of pooled male and female blood ethanol elimination rates ± one standard deviation. The number of individual determinations is given in parentheses. In comparing the two genotypes, whether naive or ethanol-fed, the values are significantly different ($p < 0.0005$). Ethanol feeding does not significantly alter the blood ethanol elimination rate in Adh^N/Adh^N animals, but does in Adh^F/Adh^F animals ($p < 0.005$). From Burnett, K.G., and Felder, M.R. Ethanol metabolism in *Peromyscus* genetically deficient in alcohol dehydrogenase. *Biochem Pharmacol,* 29:125-130, 1980. Copyright 1980 by Pergamon Press Ltd. [Elmsford, NY].)

Table 2. **ADH, Catalase, and MEOS Activities in Naive and Ethanol-Fed *P. Maniculatus****

Animal Group	ADH (units/mg liver protein)	Catalase (units/mg liver protein)	Catalase (units/mg liver microsomal protein)	MEOS (propanol + azide) (units/mg liver microsomal protein)	MEOS (propanol + azide) (units/0.25 g liver)
Adh^{F}/Adh^{F} (Naive)	0.22 ± 0.07	5.75 ± 1.54	0.38 ± 0.14	8.46 ± 3.10	19.12 ± 7.03
Adh^{F}/Adh^{F} (Ethanol-fed)	0.28 ± 0.12	8.08 ± 2.09	0.38 ± 0.10	25.46 ± 6.60	64.22 ± 11.19
Adh^{N}/Adh^{N} (Naive)	0	8.02 ± 3.52	0.46 ± 0.31	19.35 ± 5.38	69.33 ± 21.42
Adh^{N}/Adh^{N} (Ethanol-fed)	0	7.63 ± 4.00	0.55 ± 0.27	55.57 ± 7.53	230.98 ± 90.29

*No significant difference was observed between males and females for these activities. Values presented here are the averages ± S.D. of 6 to 10 animals. Ethanol feeding does not significantly alter ADH activity in Adh^{F}/Adh^{F} animals, nor is catalase activity significantly different in any of the four groups of animals. MEOS activity is significantly different ($p < 0.0005$) between naive Adh^{N}/Adh^{N} and Adh^{F}/Adh^{F} animals. Ethanol feeding significantly alters MEOS values in both strains ($p < 0.0005$). From Burnett, K.G., and Felder, M.R. Ethanol metabolism in *Peromyscus* genetically deficient in alcohol dehydrogenase. *Biochem Pharmacol*, 29:125-130, 1980. Copyright 1980 by Pergamon Press Ltd. [Elmsford, NY].)

Table 3. Measurements of Selected Microsomal Components of Naive and Ethanol-Fed *P. Maniculatus* from Table 2

Animal Group	NADPH-cytochrome c reductase (units/mg microsomal protein)	Cytochrome P-450 (nmoles/mg microsomal protein)	Phospholipid (μmoles/mg microsomal protein)	NADPH-oxidizing activity* (ΔA_{340}/5min/mg microsomal protein)
Adh^F/Adh^F (Naive)	1.65 ± 0.57	0.34 ± 0.18	3.00 ± 0.69	0.54 ± 0.28
Adh^F/Adh^F (Ethanol-fed)	1.32 ± 0.21	0.42 ± 0.26	2.95 ± 0.43	0.48 ± 0.18
Adh^N/Adh^N (Naive)	1.40 ± 0.28	0.31 ± 0.11	2.52 ± 0.74	0.30 ± 0.06
Adh^N/Adh^N (Ethanol-fed)	2.12 ± 0.56	0.26 ± 0.05	2.56 ± 0.30	0.21 ± 0.03

* The value for NADPH oxidase in ethanol-fed Adh^N/Adh^N mice is significantly different from the naive Adh^F/Adh^F values ($p<0.1$) and from ethanol-fed Adh^F/Adh^F ($p<0.05$) and naive Adh^N/Adh^N($p<0.05$). All other component values are not significantly different in the four groups of animals. Values are average ± S.D. of 6 to 10 animals. From Burnett, K.G., and Felder, M.R. Ethanol metabolism in *Peromyscus* genetically deficient in alcohol dehydrogenase. *Biochem Pharmacol,* 29:125-130, 1980. Copyright 1980 by Pergamon Press Ltd. [Elmsford, NY].)

Reconstitution of the Microsomal Ethanol Oxidizing System: Qualitative Changes of Cytochrome P-450 After Chronic Ethanol Consumption*

Kunihiko Ohnishi, Fumio Nomura, and
Charles S. Lieber

The microsomal ethanol oxidizing system (MEOS) has characteristics similar to those of the mixed function oxidases (Lieber and DeCarli 1970*a*), including reconstitution with cytochrome P-450, NADPH-cytochrome c reductase, and phospholipid (figure 1). Cytochrome P-450 was partially purified by the method of Imai and Sato (1974) from liver microsomes of ethanol-treated rats (Lieber and DeCarli 1970*b*), and NADPH-cytochrome c reductase was purified by the method of Yasukochi and Masters (1976) using phenobarbital-treated rats (Ohnishi and Lieber 1978). When the two microsomal components and dilauroyl L-3-phosphatidyl choline were combined, the rate of the acetaldehyde production was strikingly increased, whereas each of the components did not oxidize ethanol by itself.

The acetaldehyde production was dependent on the concentration of cytochrome P-450 and NADPH-cytochrome c reductase. The reconstituted ethanol oxidizing activity was not inhibited by either catalase inhibitor 1 mM sodium azide or alcohol dehydrogenase inhibitor 2 mM pyrazole. The characteristics of this reconstituted ethanol oxidizing system were similar to those of the intact microsomal ethanol oxidizing system (optimal pH, apparent Km value for ethanol, requirement for oxygen and NADPH, partial inhibition of the activity by CO) (Ohnishi and Lieber 1977).

Evidence also was found that indicates that ethanol consumption induces a specific form of cytochrome P-450. Liver microsomes were prepared from ethanol-treated, control, phenobarbital-treated, and 3-methylcholanthrene-treated rats. The CO-reduced difference spectral maxima of cytochrome P-450 shifted toward 451 nm (figure 2). Furthermore, when liver microsomes prepared from ethanol-treated, control, phenobarbital-treated, and 3-methylcholanthrene-treated

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NOTE: Figures and tables appear at end of paper.

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rats were subjected to protein electrophoresis on sodium dodecyl sulfate-polyacrylamide gel with continuous buffer system (Welton and Aust 1974), 3,3′, 5,5′-tetramethyl benzidine staining of these gels in the presence of H_2O_2 showed three distinct hemoproteins whose molecular weights ranged between approximately 50,000 and 60,000. Chronic ethanol feeding increased two hemoproteins (nos. 1 and 2). One hemoprotein (no. 2) is distinct from hemoprotein (no. 1) induced by phenobarbital treatment and hemoprotein (no. 3) induced by 3-methylcholanthrene treatment (see figure 3). There were also changes in microsomal proteins. Liver microsomes prepared from ethanol-treated and control rats were subjected to protein electrophoresis on sodium dodecyl sulfate-polyacrylamide gel with discontinuous buffer system (Laemmli 1970). These gels were scanned (figures 4 and 5).

Major differences were in the distribution of the protein constituents with an apparent molecular weight of approximately 50,000, which is the cytochrome P-450 region. After chronic ethanol feeding, a protein with an apparent molecular weight of 53,400 (band no. 3) strikingly increased and a protein with an apparent molecular weight of 52,000 (band no. 2) also increased. Judging from the relative location of the protein and hemoprotein bands (within the 50,000 and 60,000 molecular weight region) and from the relative inducibility of each protein and hemoprotein band by different treatments, the increase of proteins with apparent molecular weights of 52,000 and 53,400 may be reflecting the increase of apoproteins of hemoprotein no. 2 and hemoprotein no. 3, respectively, after chronic ethanol feeding.

To delineate a role of these qualitative changes of cytochrome P-450 in the adaptive increase in the activity of the microsomal ethanol oxidizing system, the capacity of cytochrome P-450 from ethanol-treated rats or controls to promote ethanol oxidation was compared in the reconstituted system, in the presence of excess NADPH-cytochrome c reductase and dilauroyl L-3-phosphatidyl choline. The partially purified cytochrome P-450 from ethanol-treated rats was about twice as active for ethanol oxidation as for the control preparation (figure 6).

Although it has been shown that the activity of the microsomal ethanol oxidizing system can be reconstituted with cytochrome P-450, NADPH-cytochrome c reductase, and a synthetic phospholipid, all free of catalase and alcohol dehydrogenase (Miwa et al. 1978; Ohnishi and Lieber 1977), the biochemical steps involved in this pathway are not completely resolved as yet. Superoxide has been incriminated as a form of active oxygen in the mixed function oxidases (Strobel and Coon 1971). Hydroxyl radical has been reported to be generated by the microsomes in the presence of oxygen and NADPH (Cohen and Cederbaum 1979), and it has been implicated in the activity of the microsomal ethanol oxidizing system in naive rats (Cederbaum et al. 1977). After chronic alcohol consumption, the activity of the microsomal ethanol oxidizing system is strikingly increased (Lieber and DeCarli 1970*a*). Therefore, we investigated whether superoxide and hydroxyl radicals are required for the activity of the intact and reconstituted MEOS and whether these compounds are responsible for the adaptive increase of MEOS activity after chronic alcohol consumption.

To answer these questions, experiments were designed utilizing the reconstituted MEOS and intact MEOS. Cytochrome P-450 and NADPH-cytochrome c reductase were partially purified as described in a previous paper (Ohnishi and Lieber 1978). Using the reconstituted system consisting of cytochrome P-450 (0.036 nmole/ml), NADPH-cytochrome c reductase (200 units/ml), and dilauroyl L-3-phosphatidyl choline (5μg/ml), we tested the effect of superoxide dismutase (scavenger for 0_2^{-}.) on the activity of the reconstituted MEOS. It was found that superoxide dismutase did not inhibit the activity of the reconstituted MEOS. By contrast, at the concentration used, the superoxide dismutase completely abolished the xanthine-xanthine oxidase-supported ethanol oxidizing activity which is known to involve O_2^{-}. (table 1). These data suggest that superoxide is not involved in the activity of the reconstituted MEOS, although it is responsible for the activity of the xanthine-xanthine oxidase-supported ethanol oxidation. Recently, it was reported that superoxide anions are necessary for the P-450 dependent oxidation of ethanol in reconstituted membrane vesicles (Ingelman-Sundberg and Johansson 1981). In their system, a phenobarbital inducible form of rabbit liver microsomal cytochrome P-450 was used instead of an ethanol inducible form of cytochrome P-450. Possible differences between the phenobarbital and ethanol inducible forms in terms of the mechanism of ethanol oxidation remain to be studied.

To determine whether the hydroxyl radical is required for the activity of the reconstituted MEOS, we tested the effect of known hydroxyl radical scavengers. These hydroxyl radical scavengers (mannitol 100 mM, benzoate 100 mM, dimethylsulfoxide 50 mM and thiourea 20 mM) reduced the activity of the reconstituted MEOS by 67 percent, 48 percent, 76 percent, and 66 percent, respectively. By contrast, urea (100 mM, a membrane-disrupting agent) reduced the activity of the reconstituted MEOS by only 13 percent (table 2). It is very likely that the hydroxyl radical is, at least in part, involved in the activity of the reconstituted MEOS. Alternatively, scavengers could simply act as substrates for cytochrome P-450 and compete with ethanol for "active oxygen" generated. Recently, it has been shown that microsomes actively generate the corresponding hydrocarbon gas from each hydroxyl radical scavenger (3-thiomethylpropanol, 2-keto-4-thiomethylbutyric acid, dimethylsulfoxide) in the presence of NADPH and that the production of hydrocarbon gas was inhibited by the addition of ethanol (Cohen and Cederbaum 1980).

Next we wondered whether the hydroxyl radical was also involved in the adaptive increase of MEOS after chronic alcohol consumption. To that effect, microsomes were prepared from ethanol-fed rats and their pair-fed controls (Lieber and DeCarli 1970*a*), and the effects of the hydroxyl radical scavengers on MEOS activity in these two groups were tested. As shown in table 3, chronic alcohol feeding resulted in a striking increase in MEOS activity. It was found that hydroxyl radical scavengers inhibited the activity of MEOS in both ethanol-fed rats and their pair-fed controls. Indeed, the amount of MEOS activity in ethanol-fed rats inhibited by scavengers was more than the baseline levels found in controls. This may indicate that hydroxyl radicals are related to the enhancement of MEOS activity after chronic alcohol consumption. However, the difference between the MEOS activity in ethanol-fed rats and that in their pair-fed controls remained in the presence of the scavengers, suggesting that other mechanisms are also responsible for the increase in MEOS activity.

Conclusions

The microsomal ethanol oxidizing system (MEOS) can be reconstituted with cytochrome P-450, NADPH-cytochrome c reductase, and phospholipid.

Chronic ethanol consumption results in an increase of cytochrome P-450 with

- Absorption maximum of CO-difference spectrum shifted to a higher wavelength.
- Apoprotein of apparent molecular weight of 53,400.
- Greater activity in ethanol oxidation.

Hydroxyl radical may be, at least in part, responsible for the activity of the reconstituted and intact MEOS and the adaptive increase in MEOS activity after chronic alcohol consumption, but other, as yet unidentified, mechanisms are probable.

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Figure 1. Ethanol Oxidation by the Reconstituted System Containing Cytochrome P-450 (0.045 nmol/ml), NADPH-Cytochrome c Reductase (200 units/ml), and Dilauroyl L-3-Phosphatidyl Choline (5 μg/ml)

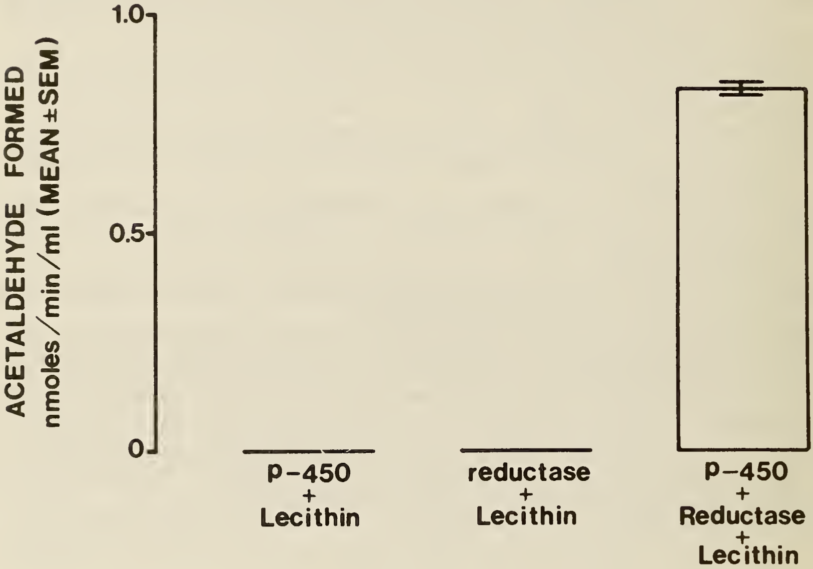
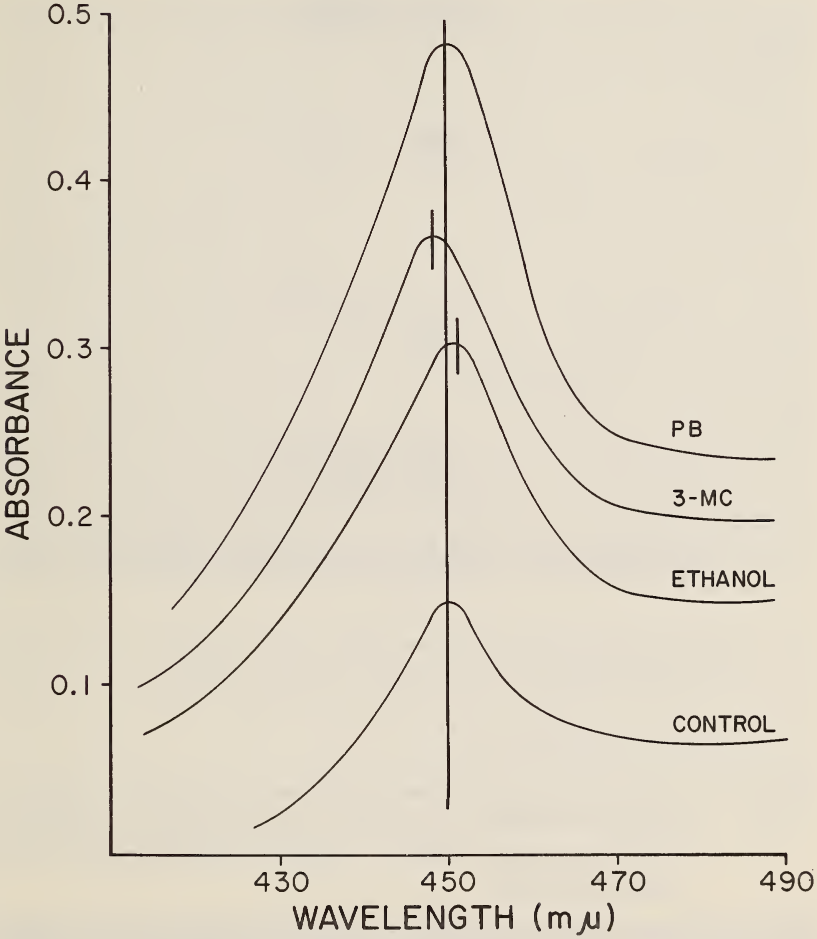
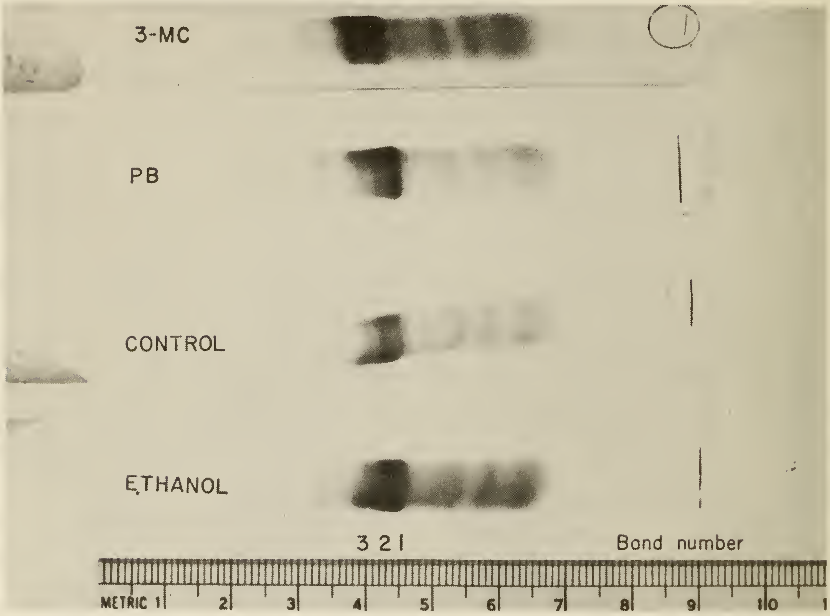


Figure 2. Effect of Various Inducers (Phenobarbital, 3-Methylcholanthrene, or Ethanol, on the CO-Difference Spectra of Hepatic Microsomal Cytochrome P-450



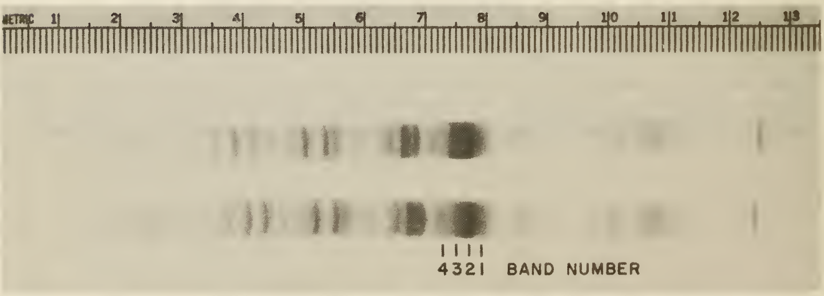
Male rats were treated with phenobarbital (80 mg/kg intraperitoneally 4 days), 3-methylcholanthrene (20 mg/kg intraperitoneally 2 days), or ethanol liquid diet (8 weeks). The protein concentration of the microsomal suspensions was 1.0 mg/ml.

Figure 3. Microsomal Hemoprotein Profiles of Control, Ethanol-Fed, Phenobarbital-Treated, and 3-Methylcholanthrene-Treated Rats



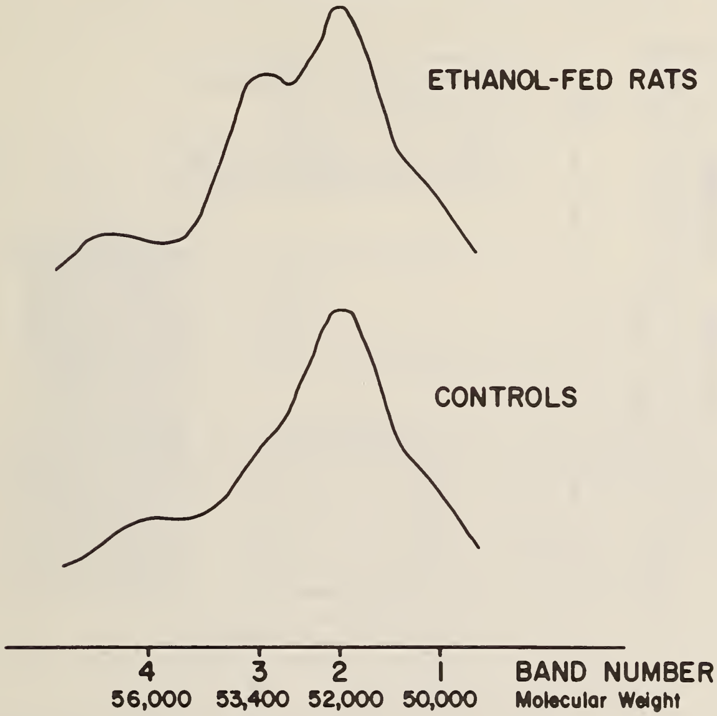
Pair-feeding of rats with liquid diets containing ethanol or isocaloric carbohydrates and phenobarbital (PB) and 3-methylcholanthrene (MC) treatments of rats were carried out as described in figure 2. Microsomal protein (120 μg) was applied to each 5.0 percent polyacrylamide gel. After electrophoresis, gels were stained by 3,3′, 5,5′-tetramethylbezidine plus H_2O_2. The apparent molecular weights of band numbers 1, 2, and 3 are 47,000, 50,000, and 56,000, respectively.

Figure 4. Coomassie Blue Protein Profiles Obtained by Electrophoresis of Liver Microsomes From Control and Ethanol-Fed Rats on 0.1 Percent Sodium Dodecyl Sulfate-Polyacrylamide Gels



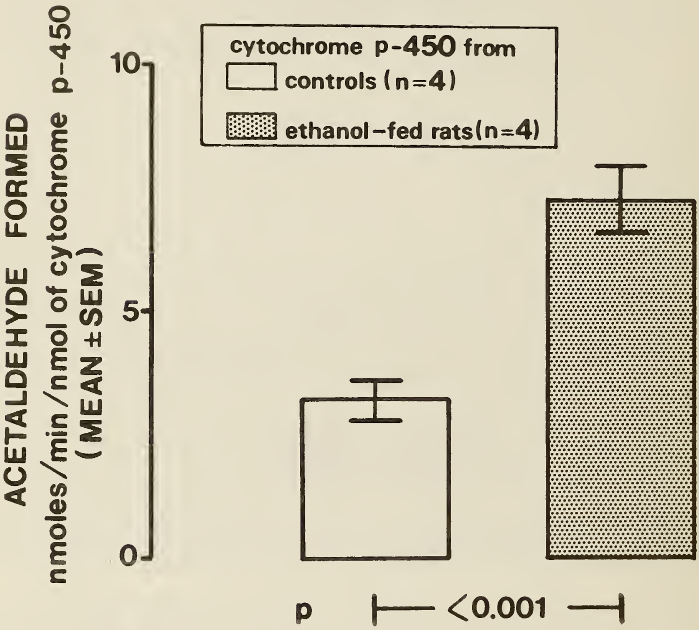
Microsomal protein was electrophoresced by the method of Laemmli (1970) with 7.5 percent separating gel after applying 40 μg of protein to each gel. Protein profiles are from hepatic microsomes of ethanol-fed (above) and control (below) rats. Band number 3 indicates the position to which a protein of molecular weight of 53,400 would migrate. The area of bands 1 to 4 was scanned (see figure 5).

Figure 5. Scans of the 50,000 Molecular Weight Region of the Gels Shown in Figure 4



Relative density of each band at 550 nm is plotted on the ordinate. Peak heights of band number 2 in both gels are automatically set to the same height. Migration distance is on the abscissa. The upper scan was obtained with microsomes from ethanol-fed and the lower from control rats. Bands 1, 2, 3, and 4 have the apparent molecular weights shown underneath the band number.

Figure 6. **Ethanol Oxidation by the Microsomal Ethanol Oxidizing System Reconstituted With Cytochrome P-450 From Ethanol-Fed or Control Rats**



The reconstituted system consisted of cytochrome P-450 (0.045 nmol/ml) from ethanol-fed or control rats, NADPH-cytochrome c reductase (200 units/ml, and dilauroyl L-3-phosphatidyl choline (5 μg/ml).

Table 1. Effect of Superoxide Dismutase on the Activity of the Reconstituted Ethanol Oxidizing System and Ethanol Oxidation by Xanthine-Xanthine Oxidase

	Acetaldehyde formed (nmole/ml/min)
P-450 + reductase + lecithin + NADPH	0.93
P-450 + reductase + lecithin + NADPH + superoxide dismutase	0.93
Xanthine oxidase + xanthine	1.29
Xanthine oxidase + xanthine + superoxide dismutase	0.05
P-450 + reductase + lecithin + NADPH xanthine oxidase + xanthine	2.09
P-450 + reductase + lecithin + NADPH xanthine oxidase + xanthine + superoxide dismutase	0.93

Notes: The reconstituted ethanol oxidizing system consisted of cytochrome P-450 (0.036 nmol), NADPH-cytochrome c reductase (200 units), and dilauroyl L-3-phosphatidyl choline (5μg). The incubations were carried out at 37°C in 1 ml of medium containing 50 μmol of ethanol, 6 μmol of $MgCl_2$, 1 μmol of Na_2-EDTA, 0.4 μmol of NADPH, and 100 μmol of potassium phosphate buffer (pH 7.4) in a 25-ml glass bottle designed for use in a Perkin-Elmer F-40 gas liquid chromatograph. Ethanol oxidation by xanthine-xanthine oxidase system was measured in a reaction mixture consisting of xanthine oxidase (0.0032 units), 0.2 μmol of xanthine, 50 μmol of ethanol, 6 μmol of $MgCl_2$, 1 μmol of Na_2-EDTA, and 100 μmol of potassium phosphate buffer (pH 7.4) at 37°C. The reaction was initiated by adding ethanol or ethanol plus xanthine oxidase and carried out in the closed system. The reaction was stopped at 0, 10, and 20 min by adding 0.2 ml of 35 percent perchloric acid containing (per ml) 100 μmol of thiourea. Where indicated, superoxide dismutase was added at the concentration of 50 μg/ml. Values represent means of two different experiments (in duplicate).

Table 2. Effect of Hydroxyl Radical Scavengers on Ethanol Oxidation Catalyzed by the Reconstituted System

	Concentration M	Ethanol oxidation (percent of control)
Control		100
Mannitol	1×10^{-1}	33.1
Benzoate	1×10^{-1}	52.3
Dimethylsulfoxide	1.25×10^{-2}	79
Dimethylsulfoxide	2.5×10^{-2}	56
Dimethylsulfoxide	5×10^{-2}	24.3
Thiourea	2×10^{-2}	34
Urea	1×10^{-1}	86.7

Note: The reconstituted system consisted of cytochrome P-450 (0.036 nmol/ml), NADPH-cytochrome c reductase (200 units/ml), and dilauroyl-L-3-phosphatidyl choline (5 μg/ml). The incubations were carried out as described in table 1. When the effects of hydroxyl radical scavengers were studied, the scavengers were included in the incubation medium. Values represent means of two experiments (in duplicate).

Table 3. Effect of Hydroxyl Radical Scavengers on Ethanol Oxidation by Microsomal Preparations From Ethanol-Fed Rats and Their Pair-Fed Controls

	Microsomal Preparations			
	Ethanol-fed		Controls	
Additions to Reaction Mixture	Specific Activity (nmol/acetaldehyde/min/mg protein	Relative Activity (percent)	Specific Activity (nmol/acetaldehyde/min/mg protein	Relative Activity (percent)
None	15.9 ± 0.97	100	5.8 ± 0.64	100
Dimethylsulfoxide (50mM)	4.5 ± 0.13	28	1.4 ± 0.05	24
Mannitol (100mM)	5.7 ± 0.30	36	1.8 ± 0.04	31
Benzoate (100mM)	7.6 ± 0.46	48	1.5 ± 0.05	26
Thiourea (20mM)	5.9 ± 0.23	37	1.6 ± 0.19	28

Note: The reaction mixtures (in a final volume of 1.0 ml), were incubated at 37°C and contained 1 mg of microsomal protein, 1 μmol of sodium azide, 100 μmol of potassium phosphate buffer (pH 7.4), 6 μmol of $MgCl_2$, 1 μmol of Na_2-EDTA, and 0.4 μmol of NADPH, and 50 μmol of ethanol. The reaction was initiated by adding ethanol and stopped at 0, 5, and 10 min by adding 0.2 ml of 35 percent perchloric acid containing (per ml) 100 μmol of thiourea. When the effect of hydroxyl radical scavengers was studied, the respective compound was included in the incubation medium. Values represent means ± S.E. of four experiments.

Further Evidence for the Role of Hydroxyl Radicals in the Microsomal Oxidation of Alcohols; Effect of the Free-Radical, Spin-Trapping Agent, 5,5-Dimethyl-1-Pyrroline-1-Oxide*

Arthur I. Cederbaum and Gerald Cohen

Introduction

The molecular mechanism underlying the oxidation of alcohols by liver microsomes (Lieber and DeCarli 1970; Teschke et al. 1977) is still not understood. Alcohols react with hydroxyl radicals (·OH) to produce the corresponding aldehydes (Anbar and Neta 1967). Recent experiments from our laboratory suggested the possibility that microsomal oxidation of alcohols represents, at least in part, the interaction of alcohols with hydroxyl radicals (·OH) generated from microsomal electron transfer (Cederbaum et al. 1977, 1978; Cederbaum, Dicker, Rubin, and Cohen 1979). The addition of a series of ·OH scavengers inhibited microsomal oxidation of ethanol and 1-butanol. Kinetic experiments indicated that the inhibition by the scavengers was competitive with respect to ethanol. The inhibition by the scavengers was specific because catalase-dependent oxidation of ethanol, microsomal drug metabolism, and microsomal electron transfer were not affected by concentrations of the scavengers which blocked the oxidation of ethanol and 1-butanol. Chemical evidence for the production of ·OH by microsomes was presented (Cohen and Cederbaum 1979, 1980). Experiments with azide and external addition of H_2O_2 led to the suggestion that H_2O_2 serves as a precursor of ·OH in the microsomal system (Cederbaum et al. 1978; Cohen and Cederbaum 1980). The addition of an iron-chelate (iron-EDTA) increased microsomal oxidation of alcohols (Cederbaum et al. 1980). Iron-chelates are known to increase ·OH production in biological systems (Halliwell 1978; McCord and Day 1978). Taken as a whole, these results suggested that the

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NOTE: Figures appear at end of paper.

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molecular mechanism underlying the oxidation of alcohols by liver microsomes reflects the ability of the alcohols to interact with ·OH generated from microsomal electron transfer. A scheme summarizing these results is shown in figure 1. More recent experiments indicated that ·OH can be generated by reconstituted systems containing cytochrome P-450 purified from phenobarbital-treated rats (Cederbaum, Miwa, Cohen, and Lu 1979) and that ·OH plays a role in ethanol oxidation by purified reconstituted systems (Cederbaum, Miwa, Cohen, and Lu 1979; Ohnishi and Lieber 1978).

In these studies, it has not been possible to definitively distinguish ·OH from other possible free radicals, such as alkoxy or peroxy radicals, by chemical means.

Recent electron spin resonance (ESR) studies by Lai and Piette (1977, 1978) showed the presence of a free radical generated by rat liver microsomes during electron transport initiated by NADPH. The radical was trapped and thereby detected with two spin-trapping agents, namely, 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) and phenyl-*tert*-butyl-nitrone. The hyperfine splitting constants of the ESR spectra indicated that the radical produced by the microsomes was the hydroxyl radical.

The above ESR studies provide a convenient opportunity to further evaluate the role of ·OH in microsomal oxidation of alcohols. Because the substance that is spin-trapped during microsomal electron transfer has been identified as ·OH, it follows that DMPO might be an effective inhibitor of the microsomal oxidation of alcohols. If this were to prove true, then other free radicals would in all probability be excluded as the reactive species generated by microsomes and stronger evidence would be provided for the role of ·OH in the microsomal metabolism of alcohols, dimethylsulfoxide, methional, and KTBA. In the current study, the effect of DMPO on microsomal oxidation of ethanol and associated microsomal functions was evaluated. Full details and methods are reported elsewhere (Cederbaum and Cohen in press).

Results

Effects of DMPO on the Microsomal Metabolism of Alcohols and 2-Keto-4-Thiomethyl Butyric Acid (KTBA)

In the presence of 0.5 mM azide, which was added to inhibit catalase, DMPO inhibited the oxidation of both ethanol and 1-butanol by rat liver microsomes (figure 2). Progressive inhibition was observed at increasing concentrations of DMPO. Inhibition was significant at concentrations of 3.3 mM (ethanol oxidation) or 6.7 mM DMPO (1-butanol oxidation). At higher concentrations of DMPO, greater inhibition was observed with ethanol than with 1-butanol. Control experiments indicated that DMPO had no effect on the NADPH-generating system (viz., glucose-6-phosphate dehydrogenase activity) nor on the recovery of standard amounts of acetaldehyde.

Ethanol oxidation was studied in greater detail (figure 3). In the absence of DMPO, the amount of acetaldehyde produced increased as the concentration of ethanol was raised (legend to figure 3). DMPO was a more effective inhibitor at lower concentrations of ethanol. A Lineweaver-Burk plot of the control rates of ethanol oxidation in the absence of DMPO was linear with a V of 8.5 nmol/min/mg protein and a K_m of 13 mM. DMPO, at concentrations of 1, 3.3, 6.7, 10, and 30 mM, increased the K_m for ethanol to values of 20, 33, 40, 50, and 67 mM, respectively, while V values were 8.5, 8.3, 7.5, 6.9, and 6.0 nmol/min/mg protein, respectively. At lower concentrations, DMPO appeared to inhibit ethanol oxidation competitively; at higher concentrations, deviations from conventional competitive kinetics were evident.

Ethanol oxidation by microsomes can also be catalyzed by the peroxidatic activity of catalase, providing that azide (catalase inhibitor) is omitted. Catalase-dependent ethanol oxidation in the absence of azide is readily observed when 3 mM xanthine plus 0.1 unit xanthine oxidase serves as a source of H_2O_2 in place of the NADPH-generating system. Under these conditions, ethanol was oxidized at a rate of 8.1 nmol/min/mg microsomal protein (figure 4). This catalase-dependent activity was not affected by DMPO (figure 4). NADPH-dependent oxidation of ethanol in the absence of azide also was shown to involve catalase; the oxidation of NADPH generates H_2O_2 for the peroxidatic activity of catalase. This pathway of ethanol oxidation also was not affected by DMPO (figure 4). These results indicate that DMPO does not inhibit the catalase-dependent oxidation of ethanol.

KTBA produces ethylene gas on reaction with hydroxyl radicals (Cohen and Cederbaum 1979, in press). When KTBA replaced ethanol and 1-butanol as a substrate for microsomes, ethylene production was progressively inhibited by increasing concentrations of DMPO (figure 5). Inhibition was significant at DMPO concentrations as low as 3.3 mM. Thus, metabolism of aliphatic alcohols as well as an independent chemical scavenger for ·OH, namely, KTBA, was inhibited by DMPO.

Effect of DMPO on the Stimulation of Microsomal Oxidation of Ethanol by Iron-EDTA

Iron-chelates stimulate production of ·OH in biological systems (Halliwell 1978; McCord and Day 1978). Iron-EDTA stimulated microsomal oxidation of ethanol by two- to threefold (Cederbaum et al. 1980). Iron-EDTA also increases the ESR signal for ·OH when DMPO is added to microsomes (Lai and Piette 1978). Therefore, it is to be expected that DMPO would inhibit the stimulation of ethanol oxidation by iron-EDTA. An iron-EDTA chelate was prepared with EDTA present at twice the molar concentration of iron. Figure 6 shows that at iron concentrations of 8.3 and 50 μM, the rates of ethanol oxidation were increased from 7.86 to 15.79 and 23.57 nmol/min/mg microsomal protein, respectively, representing increases of 101 percent and 200 percent, respectively. DMPO was effective in inhibiting ethanol oxidation in the presence of iron-EDTA in much the same manner as it inhibited control rates in the absence of iron-EDTA. At the higher concentration of iron-EDTA (50 μM), nearly the entire increase in the ethanol oxidation rate was abolished by 30 mM DMPO.

Effect of DMPO on Microsomal Drug Metabolism

Concentrations of DMPO that strongly inhibited the azide-insensitive rate of ethanol oxidation had no effect on the activities of aminopyrine demethylase or aniline hydroxylase (figure 7). Initial rates of NADPH-dependent oxygen uptake also were not affected by DMPO: the control rate in the absence of DMPO was 27.6 natom/min/mg microsomal protein, while rates in the presence of 3.3, 6.7, 10, and 30 mM DMPO were 24.6, 25.3, 25.5, and 24.6 natom/min/mg protein, respectively. In a similar manner, DMPO had no effect on NADPH-dependent cytochrome *c* reductase activity; the control rate was 36.7 nmol cytochrome *c* reduced/min/mg protein, while rates in the presence of 3.3, 6.7, 10, and 30 mM DMPO were 36.5, 32.0, 35.9, and 32.2 nmol/min/mg protein, respectively. These observations rule out the possibility of nonspecific interactions of DMPO with microsomes.

Effect of Chelex 100 or DETAPAC on Ethanol Oxidation by Microsomes

Passage of the water, KCl, phosphate, and pyrophosphate solutions through a column of Chelex 100 resin did not alter the rate of ethanol oxidation (figure 8). No significant effect of Chelex pretreatment was noted previously for the microsomal production of ethylene from KTBA (Cohen and Cederbaum 1979). Other investigators have shown that the chelating agent, DETAPAC, will block production of ·OH in the metal-catalyzed Haber-Weiss reaction (Buettner and Oberley 1978; Halliwell 1978). However, in our experiments, 200 μM DETAPAC produced only a small suppression of microsomal oxidation of ethanol (figure 8).

Discussion

Previous studies by others (Lai and Piette 1977, 1978) used DMPO to detect a free radical, identified as the hydroxyl radical, generated by microsomes. Results in the current report link the oxidation of alcohols by microsomes to the ESR studies and identify the radical species responsible for ethanol oxidation as the hydroxyl radical. Consistent with this suggestion are the observations that allow a comparison of the radical species responsible for ethanol oxidation to the radical trapped by DMPO: (a) both are stimulated by iron-EDTA (Lai and Piette 1977, 1978; figure 6); (b) inhibition of ethanol oxidation by DMPO exhibits competitive aspects (figure 3), as does inhibition by other free radical scavengers (Cederbaum et al. 1977, 1978; Cohen and Cederbaum 1980); (c) ethanol blocks the generation of the DMPO·OH ESR signal by microsomes (Lai and Piette 1977; Saprin and Piette 1977); (d) DMPO and other free-radical scavengers (viz., mannitol, benzoate, dimethylsulfoxide, KTBA) inhibit the azide-insensitive pathway of ethanol oxidation selectively without affecting the microsomal metabolism of aminopyrine or aniline, or partial reactions involved in microsomal electron transfer, or the catalase-dependent pathway of ethanol oxidation (Cederbaum et al. 1977, 1978; Cohen and Cederbaum 1980; figures 2 and 5); (4) DMPO inhibits the metabolism of both KTBA and ethanol (figures 2, 3, and 5) while KTBA and ethanol cross-inhibit the metabolism of one another (Cohen and Cederbaum 1979, 1980).

The reactive free radical that is generated by microsomes and oxidizes alcohols and metabolizes KTBA or methional (to ethylene) and dimethylsulfoxide (to methane) appears to be derived from generated H_2O_2 (Cederbaum et al. 1978; Cohen and Cederbaum 1980). Singlet oxygen has been excluded by the observation that it does not react with alcohols. One reaction pathway for the production of ·OH is via a Fenton-type reaction between H_2O_2 and ferrous salts or ferrous chelates. A reaction with iron contaminants in the reagents of buffer salts can be excluded because pretreatment of the water and the reagents with Chelex 100 resin did not significantly affect the metabolism of ethanol or KTBA. A reaction between iron present in the microsomal preparations (e.g., adventitious iron or cytochrome P-450) is a more likely reaction pathway. It should be emphasized, however, that added exogenous H_2O_2 (presence of azide) cannot substitute for microsomal electron flow initiated by NADPH and that boiled microsomes are without significant action either when H_2O_2 is added (presence of azide) or in the presence of the NADPH-generating system. Therefore, electron flow initiated in intact microsomes by NADPH appears to be an absolute requirement. It seems likely that one of the roles of electron flow is to reduce iron to the ferrous state (e.g., cytochrome P-450 reductase) so that it might function in a Fenton-type reaction.

Another route for production of ·OH is via an iron-catalyzed Haber-Weiss pathway. However, the microsomal pathway for ethanol oxidation was not especially sensitive to DETAPAC, which blocks the iron-catalyzed Haber-Weiss reaction. These results indicate that ·OH production and metabolism of both KTBA and ethanol are intrinsic to the microsomes when electron flow is initiated by NADPH, and that a simple, nonspecific interaction between endogenous iron and H_2O_2 can be excluded. We presume that ·OH or its equivalent (e.g., ferryl radical) is bound and not released into the medium.

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Figure 1. Scheme for the Generation of ·OH and Interaction of ·OH With Various ·OH Scavenging Agents

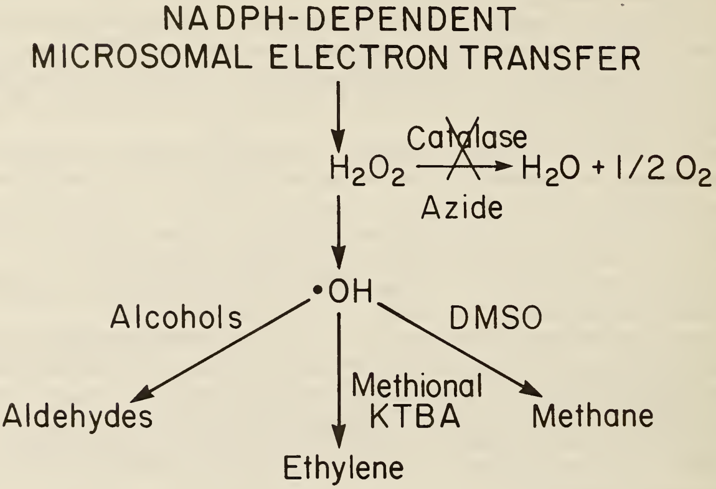
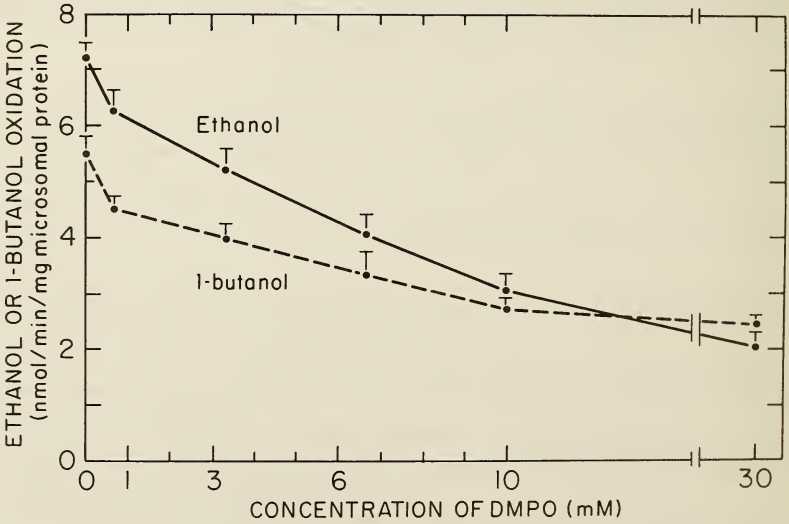
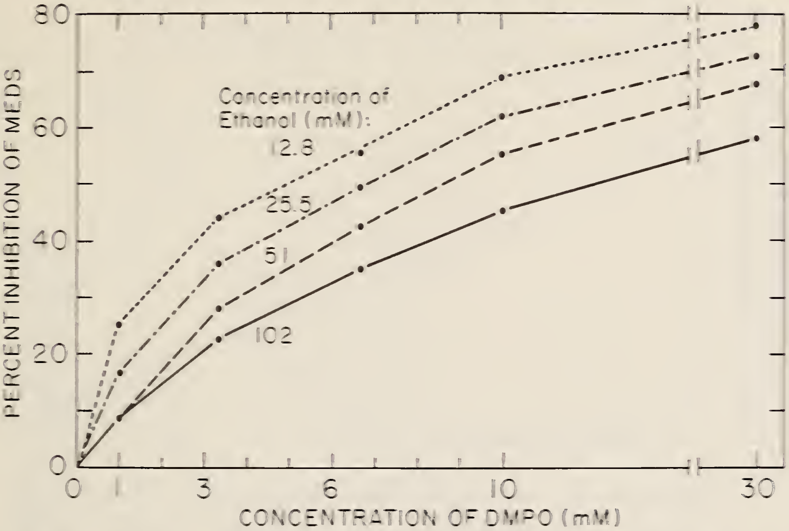


Figure 2. Effect of DMPO on Microsomal Oxidation of Ethanol (51 mM) and 1-Butanol (51 mM)



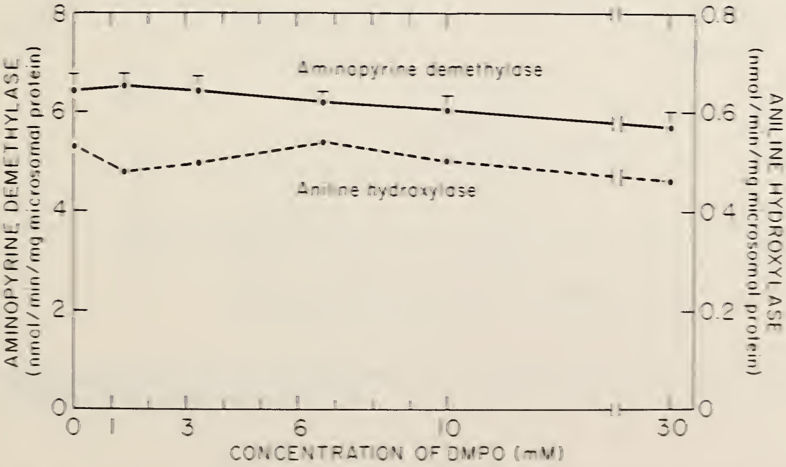
Azide was present at a final concentration of 0.5 mM.

Figure 3. Effect of Ethanol Concentration on the Inhibition of Microsomal Oxidation of Ethanol by DMPO



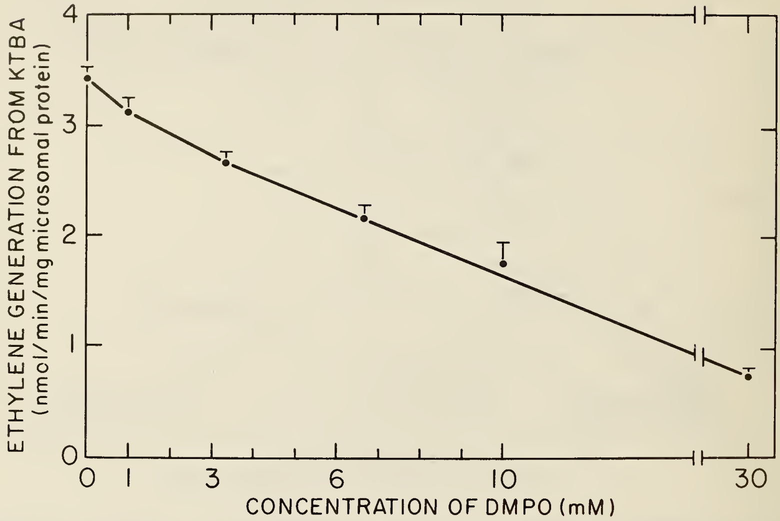
Azide (0.5 mM) was present throughout. Control rates of ethanol oxidation (nmol/min/mg microsomal protein) were 12.8 mM ethanol, 4.21±0.44; 25.5 mM ethanol, 5.19±0.61; 51 mM ethanol, 6.51±0.62; 102 mM ethanol, 7.62±0.40.

Figure 4. Effect of DMPO on Catalase—(H_2O_2)-Dependent Oxidation of Ethanol



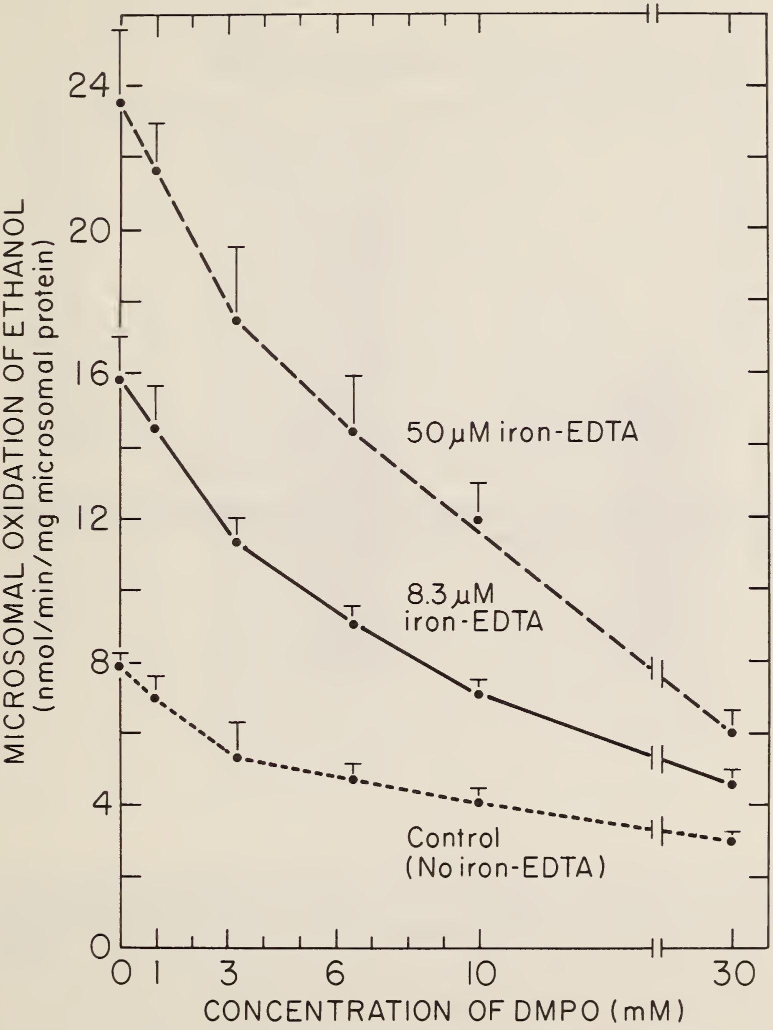
Azide was omitted to prevent inhibition of catalase. H_2O_2 was generated either by NADPH oxidation or by xanthine oxidase activity.

Figure 5. Effect of DMPO on Microsomal Generation of Ethylene Gas From KTBA (10mM)



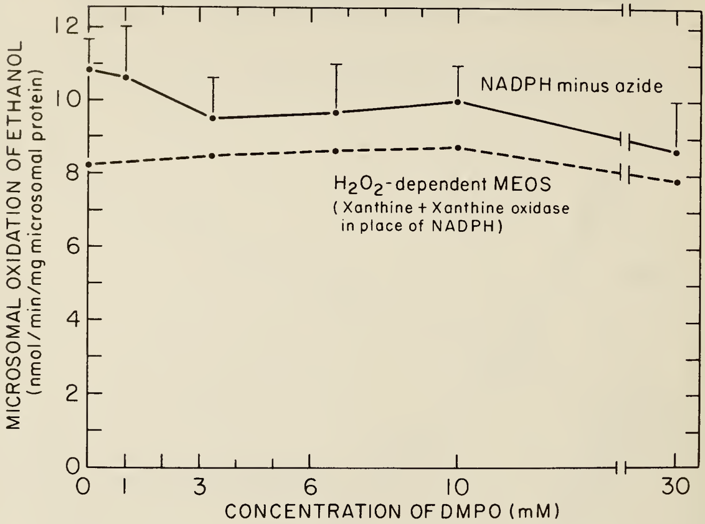
Azide was present at a final concentration of 0.5 mM.

Figure 6. Effect of DMPO on the Stimulation of Microsomal Oxidation of Ethanol by Iron-EDTA



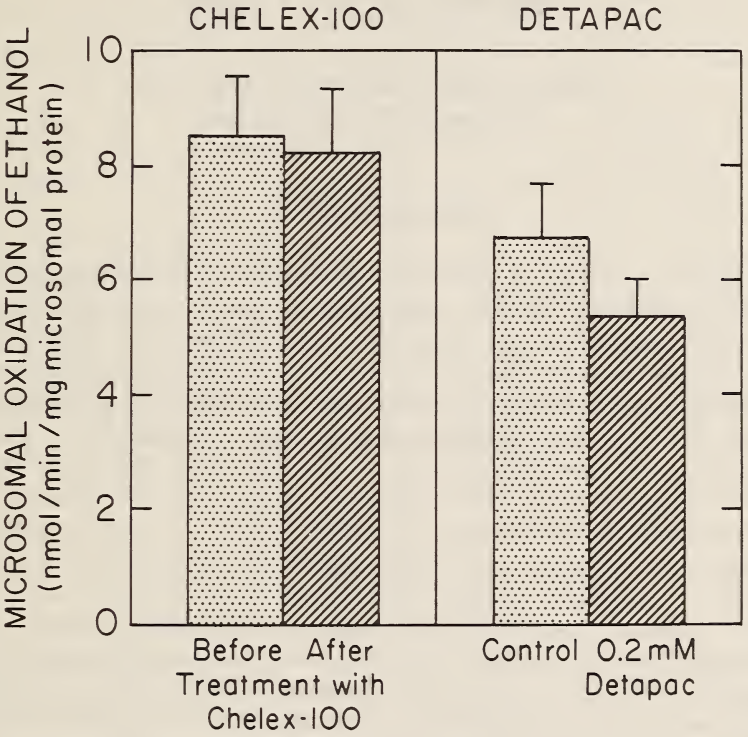
Experiments were carried out in the presence of 0.50 mM azide. EDTA was present at twice the molar concentration of iron.

Figure 7. Effect of DMPO on Microsomal Drug Metabolism



The effect of DMPO on two classical microsomal drug metabolism systems, aminopyrine demethylation and aniline hydrosylation, is shown.

Figure 8. Effect of Chelex-100 Treatment and of DETAPAC (Diethylene-Triamine Pentaacetic Acid) on Microsomal Oxidation of Ethanol



The H_2O_2 buffers and other solutions were passed through a chelex-100 resin to remove "contaminating iron." The lack of effect of chelex treatment or DETAPAC rule out a significant role for contaminating iron in microsomal oxidation of alcohols or generation of ·OH.

Relative Specificity of the Ethanol-Induced Rat Liver Cytochrome P-450 for Ethanol Oxidation Activity in Vitro

Jean-Gil Joly, Claude Hétu, and
Avinasha M. Mungikar

Introduction

The liver microsomal ethanol oxidizing system (MEOS) (Lieber and DeCarli 1968) has recently been reconstituted in vitro from highly purified mixed-function oxidase components (Miwa et al. 1978). In the rat, ethanol has been shown to induce a liver microsomal cytochrome P-450 species spectrally and catalytically different from the hemoproteins of control rats and different from those appearing after phenobarbital and 3-methylcholanthrene administration (Mungikar et al. 1978; Villeneuve et al. 1976). Although evidence was provided for the enhancement in microsomes of MEOS activity by chronic ethanol feeding to rats (Lieber and DeCarli 1970) and for the reconstitution of MEOS with partially purified cytochrome P-450 (Ohnishi and Lieber 1977), in vitro reconstitution of MEOS with highly purified cytochrome P-450 from rats chronically fed with ethanol has not yet been reported. We report that the ethanol-induced cytochrome P-450, when compared to other forms of cytochrome P-450, shows relative substrate specificity for ethanol in the reconstituted MEOS in vitro.

Experimental Procedures

Female Sprague-Dawley rats were pair-fed with an ethanol-containing (36 percent of calories) or control liquid diet (DeCarli and Lieber 1967) for 21 days. Animals receiving a laboratory chow diet were injected with phenobarbital (75 mg/kg) or 3-methylcholanthrene (25 mg/kg) for 3 days prior to sacrifice. Liver microsomal cytochrome P-450 from these four groups of rats were first purified essentially according to Guengerich (1977), with certain modifications: elution of cytochrome P-450 from hydroxyapatite was done with 110 and 150 mM phosphate buffer and only the first of the two peaks, which

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NOTE: Figure and tables appear at end of paper.

contained the major portion (70 to 80 percent) of the cytochrome P-450 eluted, was used for further purification. Most of this fraction was then found to adsorb on DEAE-cellulose, and cytochrome P-450 was eluted from this column with a 0 to 0.5 M KCl gradient. Chromatography on CM-Sephadex C-50 (Imai and Sato 1974), followed by removal of detergents using Amberlite XAD-2 treatment and calcium phosphate gel adsorption (Van der Hoeven and Coon 1974), yielded stable cytochrome P-450 preparations containing 14 to 16 nmol hemoprotein/mg of protein. NADPH-cytochrome c reductase was purified according to Yasukochi and Masters (1976) to a specific activity of 40,000 to 50,000 units/mg protein. Proteins were determined according to Lowry et al. (1951). Catalase activity (Beers and Sizer 1952) and alcohol dehydrogenase activity (Bonnichsen and Brink 1955) were not detected in either the NADPH-cytochrome c reductase or the cytochrome P-450 preparations used in this study. The determination of [1-^{14}C]ethanol oxidation by cytochrome P-450 in the reconstituted MEOS was performed according to Miwa et al. (1978) using [1,2-^{14}C]acetaldehyde diluted with water by the manufacturer to establish the acetaldehyde standard curve.

Results

The purification yielded control, ethanol, phenobarbital, and 3-methylcholanthrene cytochrome P-450 preparations containing, respectively, 16.1, 16.5, 15.0, and 14.6 nmol/mg protein. These preparations were used for assay of [1-^{14}C]ethanol oxidation activity in the reconstituted MEOS. Incubation conditions were as described (Miwa et al. 1978) except that 0.05 nmol of cytochrome P-450 was used in the assays (unless otherwise specified) and that the amounts of dilauroylphosphatidylcholine (15 μg) and of NADPH-cytochrome c reductase (12,000 units) in the assay were optimized for our preparations. Sodium azide (0.15 μmol) and pyrazole (3 μmol) were added to the incubation mixture. No activity could be observed in the absence of added reductase, whereas the dependency of MEOS on added phospholipid showed a pattern similar to that reported by others (Miwa et al. 1978).

As seen in figure 1, an absolute requirement of cytochrome P-450 is observed for the conversion of ethanol to acetaldehyde in the reconstituted MEOS. Molar activities of the various cytochrome P-450 preparations also can be seen to differ markedly. Cytochrome P-450 from ethanol-treated rats shows the highest ethanol oxidation activity, followed by the other cytochrome P-450. Control cytochrome

P-450 preparations exhibit a 4.2-fold lower activity than the ethanol-induced hemoprotein. Similar results (as shown in figure 1) were observed using two other sets of four cytochrome P-450 preparations. The average molar activities for the three sets of four cytochrome P-450 studied are given in table 1.

A difference in the apparent Km for ethanol was observed when measuring ethanol oxidation activity of the reconstituted MEOS with the ethanol-induced cytochrome P-450 preparations (5.3 mM) and the control preparations (8.0 mM). Similar apparent Km values for NADPH (62 μm and 74 μmM, respectively) were observed in the reconstituted MEOS when using ethanol and control cytochrome P-450 preparations.

Reconstituted MEOS activity with phenobarbital-induced cytochrome P-450 was relatively high. To better differentiate the ethanol-induced cytochrome P-450 MEOS activity from that mediated by the phenobarbital-induced cytochrome P-450, MEOS inhibition was studied in presence of two potential inhibitors added to the reconstituted system of ethanol oxidation. As seen in table 2, the ethanol-induced hemoprotein mediated reaction is more sensitive to inhibition by tetrahydrofurane, whereas that catalyzed by the phenobarbital-induced hemoprotein shows greater sensitivity to inhibition by metyrapone.

Discussion

Reconstitution of MEOS activity from purified essential components of microsomal mixed-function oxidases has recently closed the debate as to the nature of MEOS (Miwa et al. 1978). It therefore supports the hypothesis of the direct participation of cytochrome P-450 in the microsomal metabolism of ethanol. Our results also confirm these observations and the apparent similarity between MEOS and other cytochrome P-450 dependent mixed-function oxidases. Cytochrome P-450 preparations obtained from animals treated with various inducers of liver mixed-function oxidases are known to differ in their substrate specificity (Conney et al. 1973). Cytochrome P-450 preparations obtained from ethanol-treated rats have been shown to differ catalytically from other species of this hemoprotein (Joly et al. 1977; Mungikar et al. 1978; Villeneuve et al. 1976). We now report observations showing that highly purified ethanol-induced cytochrome P-450 also differs from other species of cytochrome P-450 with respect to its activity in the reconstituted MEOS. These results show that induction of MEOS activity following chronic ethanol administration is not only explained by an increase in microsomal cytochrome P-450 content but is also related to the appearance of at least one form of cytochrome P-450 for which ethanol is a relatively specific substrate. This conclusion had been previously reached by Ohnishi and Lieber (1977) in reconstitution experiments with slightly enriched cytochrome P-450 preparations. The significance of the finding that the reconstituted MEOS has a lower Km for ethanol in the presence of the ethanol-induced cytochrome P-450 is unclear. It may, however, be of importance with respect to ethanol metabolism in vivo. Maximal MEOS activity in vivo in alcoholics might be reached at ethanol levels lower than that needed to saturate MEOS in nonalcoholics (Lieber 1977). In conclusion, the inducibility by chronic ethanol administration of liver MEOS activity and of a cytochrome P-450 species showing relative substrate specificity for ethanol is in agreement with the hypothesis suggesting an adaptive role of MEOS in the metabolic tolerance of the alcoholic to ethanol.

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Figure 1. Titration of Ethanol Oxidation Activity of the Reconstituted MEOS by Cytochrome P-450 From Control (▲-▲), Ethanol (•-•), Phenobarbital (○-○), and 3-Methylcholanthrene-Treated (□-□) Rats

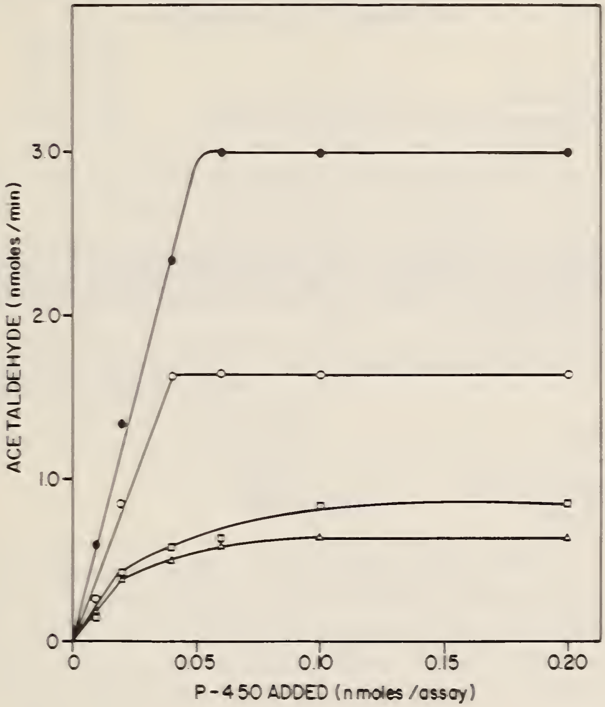

Table 1. Molar Activity of Cytochrome P-450 Preparations From Variously Treated Rats ($\overline{X}$ ± SE, n=3)

Source	Ethanol Oxidation Activity (nmol/acetaldehyde/min/nmol P-450
Control	19.9 ± 0.6
Ethanol-treated	85.4 ± 6.8
Phenobarbital-treated	62.7 ± 4.8
3-Methylcholanthrene-treated	25.6 ± 3.6

Table 2. Effect of Inhibitors on Ethanol Oxidation by Cytochrome P-450 From Ethanol (E) and Phenobarbital-Treated (PB) Animals

	Percent Inhibition of MEOS	
Inhibitor	E-P-450	PB-P-450
Tetrahydrofurane (1 mM)	84	68
Metyrapone (1 μM)	44	84

Ethanol Oxidation by Catalase in Rat Brain in Vivo

**Gerald Cohen, Pierre M. Sinet, and
Richard E. Heikkila**

Introduction

Catalase activity in the brain parenchyma, distinct from catalase in erythrocytes, has been confirmed (Gaunt and DeDuve 1976). The catalase is associated with small subcellular particles, viz., the "microperoxisomes" of brain (McKenna et al. 1976). The measurement of brain catalase and its inhibition by 3-amino-1,2,4-triazole can be used to detect both H_2O_2 formation in brain in vivo (Sinet et al. 1980) and the utilization of brain H_2O_2 for the oxidation of ethanol (Cohen et al. 1980).

Methods

Animals: Sprague-Dawley rats weighing 200 to 300 g.

Injections: Aminotriazole (1 g/kg) was injected intraperitoneally in isotonic saline. Ethanol (4 g/kg) was injected intraperitoneally in isotonic saline 30 min prior to aminotriazole. Control rats received an intraperitoneal injection of an equivalent volume of isotonic saline.

Tissue Preparation: A gentle homogenization procedure (Sinet et al. 1980) in cold isotonic saline buffered to pH 7.4 was used to avoid rupturing erythrocytes and capillaries. Only 2 percent of the erythrocytes were broken in this procedure. Samples were centrifuged at 700 × g, and the supernatant was taken for catalase assay.

Catalase Assay: The disappearance of hydrogen peroxide (1.0 mM) was measured at 0°C at pH 7.0 over 20 min. The reaction was stopped with acid, and the residual hydrogen peroxide was measured with a colorimetric method (Sinet et al. 1980).

Aminotriazole Assay: A modification of the colorimetric method of Green and Feinstein (1957) was used to measure levels of aminotriazole in brain.

NOTE: Tables appear at end of paper.

Reaction Mechanisms

Equation 1: Catalase and H_2O_2 react to form a spectroscopically distinct complex (Chance and Schonbaum 1962).

$$\text{Catalase} + H_2O_2 \longrightarrow (\text{catalase-}H_2O_2)\text{ compound I} \qquad (1)$$

Equation 2: When compound I reacts with aminotriazole, the enzymatic activity is destroyed (Margoliash et al. 1960; Tephley et al. 1961). Therefore, loss of catalase activity after exposure to aminotriazole detects the presence of hydrogen peroxide (equation 2) (Cohen and Hochstein 1965).

$$\text{Compound I} + \text{aminotriazole} \longrightarrow \text{irreversibly inhibited catalase} \qquad (2)$$

Equation 3: Ethanol reacts with compound I to give acetaldehyde (i.e., the "peroxidatic" activity of catalase) (equation 3). Reaction 3 is competitive with reaction 2. Therefore, when ethanol prevents the inactivation of catalase, it can be inferred that ethanol has served as substrate for the peroxidatic activity of catalase.

$$\text{Compound I} + \text{ethanol} \longrightarrow \text{catalase} + \text{acetaldehyde} + 2\ H_2O \qquad (3)$$

Equation 4: The "decomposition" of H_2O_2 by catalase is shown.

$$\text{Compound I} + H_2O_2 \longrightarrow \text{catalase} + O_2 + 2\ H_2O \qquad (4)$$

Results

Table 1 shows a progressive inhibition of catalase activity in two representative brain regions after an intraperitoneal injection of aminotriazole. These results indicate the presence of both H_2O_2 and compound I in rat brain in vivo (see equations 1 and 2).

Table 2 shows that ethanol prevents the inactivation of brain catalase by aminotriazole. Therefore, oxidation of ethanol to acetaldehyde by compound I is indicated (equation 3). This is true providing it is shown that ethanol has not prevented the entry of aminotriazole into brain (see ahead). The data in table 2 describe results of assays on the striatum; similar results were obtained for assays on the cortex.

Table 3 shows that ethanol does not prevent the entry of aminotriazole into brain. Therefore, ethanol acts as shown in equation 3, and the oxidation of ethanol to acetaldehyde by brain catalase and endogenously generated H_2O_2 is indicated.

Table 4 shows that erythrocyte catalase is an insignificant contaminant in the brain supernatant fraction (line 7). Therefore, it can be concluded that inhibition of brain catalase by aminotriazole reflects the presence of H_2O_2 and compound I in the brain parenchyma.

Conclusions

Ethanol oxidation by endogenous catalase and hydrogen peroxide in rat brain has been demonstrated.

Targeted neurons for acetaldehyde production are those relatively rich in microperoxisomes, namely, catecholamine cell bodies in the locus ceruleus, nucleus A_1 of the medulla, and the zona compacta of the substantia nigra (McKenna et al. 1976).

Other sites for acetaldehyde generation are the microperoxisome-containing oligodendrocytes, astrocytes, and the ependymal cells bordering the third and fourth ventricles (McKenna et al. 1976).

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Table 1. Time Course for the Effect of Aminotriazole (1 G/KG) on Catalase in Brain

	Catalase Activity (units/g tissue)					
	Prefrontal Cortex			Striatum		
Time (hr)	(mean ± S.D.)	(N)	(Percent Control)	(mean ± S.D.)	(N)	(Percent Control)
0*	5.7 ± 0.7	(12)	(100)	4.6 ± 0.8	(12)	(100)
0.5	5.3 ± 0.7	(4)	(93)	3.5 ± 0.9	(4)	(76)
1.0	2.6 ± 0.7	(8)	(46)	2.5 ± 0.4	(8)	(54)
1.5	1.6 ± 0.1	(4)	(28)	1.6 ± 0.2	(4)	(35)
5.0	0.9 ± 0.3	(4)	(16)	0.8 ± 0.4	(4)	(17)

*Zero time animals received injections of isotonic saline in place of aminotriazole.

Table 2. Effect of Ethanol (4 G/KG) on the Inhibition of Brain Catalase by Aminotriazole

	Catalase Activity (units/g tissue)			
	−Ethanol		+Ethanol	
Condition	(mean ± S.E.M.) (N)			
−Aminotriazole	4.5 ± 0.2	(16)	4.4 ± 0.3	(4)
+Aminotriazole	2.5 ± 0.1	(12)*	4.5 ± 0.4	(4)**

*$p < 0.01$ compared to animals without aminotriazole.
**$p < 0.01$ compared to animals with aminotriazole alone.

Table 3. Effect of Ethanol Pretreatment on the Level of Aminotriazole in Brain

Condition	Aminotriazole (mM)* (mean ± S.E.M.)	(N)
Control (no ethanol)	1.68 ± 0.33	(9)
Ethanol	2.02 ± 0.32	(9)

*Measurements were made at 1 hr after aminotriazole.

Table 4. "Contaminating" Erythrocyte Catalase in Brain[a]

Tissue Homogenate	Cell Count and Catalase Activity
1. Erythrocyte count (millions/g tissue)	5.8 ± 1.2
2. Calculated erythrocyte catalase (units/g tissue)[b]	0.72 ± 0.15
3. Observed total catalase in tissue (units/g)	14.9
4. Erythrocyte catalase in brain (percent of total)	5.2
Supernatant Fraction	
5. Calculated erythrocyte catalase in supernatant (units/g)[c]	0.014
6. Observed total catalase in brain supernatant (units/g)	4.5
7. Erythrocyte catalase in brain supernatant (percent of total)	0.3

[a] Analyses were carried out on the striatum.

[b] Conversion factors are 8.9×10^6 erythrocytes per mm^3 of rat blood and 1,105 units catalase per ml of rat blood (Sinet et al. 1980).

[c] Two percent of line 2 (that is, 2 percent breakage of cells during tissue homogenization procedure).

SESSION V: ACETALDEHYDE PHARMACOLOGY AND TOXICOLOGY

Hepatic Subcellular Localization of Aldehyde Dehydrogenase

Richard A. Deitrich

Liver aldehyde dehydrogenases are the enzymes responsible for the metabolism of the acetaldehyde produced by either alcohol dehydrogenase or the microsomal enzyme systems. The fact that the aldehyde dehydrogenases are reasonably successful is evidenced by the very low levels of acetaldehyde found in blood or liver in the face of relatively high rates of production of acetaldehyde from ethanol.

Most of the data on the subcellular distribution of these enzymes have been obtained from rats. There is a multitude of enzymes in rat liver. The mitochondria contain at least two enzymes—one in the matrix, which has a very low Km (μm range), and one either between the inner and outer membrane or associated with the outer membrane, with a much higher Km (mM range). Recent evidence from Smith and Lindahl (in press) raises the interesting possibility that the matrix enzyme may not be uniformly distributed either among the individual mitochondria or within the matrix of any one mitochondrion.

The microsome fraction contains a tightly bound enzyme with a Km similar to that of the outer mitochondrial membrane enzyme (Nakayasa et al. 1978). This activity may account for about 15 to 20 percent of the total.

There is recent evidence that peroxisomes may contain some aldehyde dehydrogenase. This amount probably accounts for approximately 5 percent of the total activity (Smith and Lindahl in press).

The cytosol contains at least two enzymes with relatively high Km values (mM range). The amount ranges from 5 to 20 percent of the total. These are at least two inducible enzymes; one is constitutive and is induced by phenobarbital (up to 30-fold, ϕ enzyme) in animals with the gene for this induction (Deitrich et al. 1977). Another enzyme is found in large amounts only after treatment with tetrachlorodibenzodioxin (TCDD, Υ enzyme and similar compounds, Deitrich et al. 1978) and, apparently, is the same as (or very similar to) an aldehyde dehydrogenase found in chemically induced (α enzyme) hepatic tumors (Lindahl et al. 1978).

Table 1 summarizes the current information as to location of aldehyde dehydrogenase in the liver of various species, including rat. In general, the available data suggest that the rat is a good model for

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NOTE: Table appears at end of paper.

other species, including man. The one exception to the pattern described for rat, however, is in mouse. Here, the cytosolic enzymes assume a much greater importance than they do in other species.

The functional significance of the subcellular localization of aldehyde dehydrogenase lies in the relationship of alcohol and aldehyde oxidation to the supply of NAD. When NAD is reduced to NADH by either alcohol or aldehyde dehydrogenase in the cytosol, the reducing equivalents must be moved into the mitochondria by shuttle system (Grunnet 1973). NADH produced in the mitochondrial matrix requires no shuttle system. Species in which a significant fraction of the acetaldehyde is oxidized outside the mitochondria are likely to have markedly different kinetics of alcohol and aldehyde oxidation, when such shuttle systems are rate limiting, than do those species where the bulk of acetaldehyde oxidation occurs in the mitochondrial matrix.

The differential sensitivity of aldehyde dehydrogenases toward the inhibitor disulfiram is of interest, both in the laboratory and in human therapy. It is presumed, but not demonstrated for any species except the rat (Deitrich and Erwin 1971), that inhibition of aldehyde dehydrogenase by disulfiram is irreversible. This reversal of the effects of disulfiram requires synthesis of new enzyme protein and is likely to explain the long-term effects of disulfiram. If this is true, the duration of the action of disulfiram would be a function of the half-life of aldehyde dehydrogenase, rather than of the half-life of disulfiram.

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Table 1. Location of Aldehyde Dehydrogenase, by Species

Cell fraction	Rat[1]			Mouse[2]			Horse[3]			Primate[4]		
	Percent	Km mM	Disulfiram inhibition	Percent	Km mM	Disulfiram inhibition	Percent	Km mM	Disulfiram inhibition	Percent	Km mM	Disulfiram inhibition
Mitochondria	35-80		+	15			26	2	–	60-70		
Matrix		1									11	
Intermembrane		2000									100-200	
Microsomes	26	2000	(–)				3					
Lysosomes	20	–					5					
Cytosol	4-20	1000	+(–)	85	200-800		60	70	++			
					7							
Induced ϕ	–	200	+									
Induced ϒ	–	26,000										
Tumor α	–											
Nuclei	10											

Table 1. Location of Aldehyde Dehydrogenase, by Species—Continued

Cell fraction	Man[5]			Sheep[6]			Rabbit[7]		
	Percent	Km mM	Disulfiram inhibition	Percent	Km mM	Disulfiram inhibition	Percent	Km mM	Disulfiram inhibition
Mitochondria	25	0.4 (2-3)	–	34	–	+			
Microsomes	9	3000		10					
Lysosomes	8								
Cytosol	32	0.5 10,000 (100)	++	37		++		61	
Nuclei				19					

[1] Data from Tottmar et al. 1973; Torronen and Marselos 1979; Tottmar and Marchner 1976; Deitrich and Erwin 1971.
[2] Data from Smolen et al. 1980; Smolen et al. in press.
[3] Data from Eckfeldt and Yonetani 1976.
[4] Data from Lebsack et al. 1980.
[5] Data from Koivula 1975; Greenfield and Pietruszko 1977.
[6] Data from Crow et al. 1974.
[7] Data from Duncan 1977.

Blood Acetaldehyde: Regulatory Factors During Ethanol Oxidation in Man

Mikko Salaspuro, Kai O. Lindros, and Hannu Nuutinen

The toxicity of acetaldehyde to several liver organelles such as mitochondria, endoplasmic reticulum, and microtubules is well established (Lieber et al. 1980). Furthermore, acetaldehyde has been shown to interfere with the metabolism of amines of the central nervous system (Walsh 1971). It inhibits myocardial protein synthesis (Schreiber et al. 1974) and reacts irreversibly with proteins of the human erthrocyte (Gaines et al. 1977). Until recently, however, the fate of this metabolite of ethanol has been neglected because of methodological difficulties in its measurement (Eriksson 1980). By improved methods, it has now been established that in nonalcoholic controls the acetaldehyde level of peripheral venous blood during ethanol oxidation is barely measurable, $2 < \mathrm{uM}$ (Lindros et al. 1980; Pikkarainen et al. 1979). On the other hand, there is also recent evidence supporting the elevation of blood acetaldehyde under certain conditions. According to these findings, the blood acetaldehyde level in man appears to be influenced by the rate of ethanol oxidation and by the rate of removal of acetaldehyde via hepatic acetaldehyde dehydrogenase.

Ethanol Elimination and Blood Acetaldehyde

In Chronic Alcoholics

Truitt (1971) studied blood acetaldehyde levels after oral administration of alcohol to alcoholics and nonalcoholics, but a statistically significant difference was not found. In two other earlier studies (Magrinat et al. 1973; Majchrowicz and Mendelson 1970) elevated blood levels of acetaldehyde have been found in alcoholics; but, in both studies, it was related to the amount of acetaldehyde contained as a congener in alcoholic beverage.

Korsten et al. (1975) were the first to find higher blood acetaldehyde values in alcoholics than in nonalcoholic controls after ethanol

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administration. Although these values apparently were not completely corrected for the artefactual formation of acetaldehyde during blood deproteinization, we have recently confirmed the original finding (Lindros et al. 1980).

After an intravenous infusion of ethanol (1.2 g/kg b.wt.) to nonalcoholic controls, barely detectable acetaldehyde levels (0.5 to 1μM) were observed in the early phase of the experiments, coinciding with the peak blood ethanol; but, in general, the blood acetaldehyde levels in the controls were below the detection limit (0.5μM) (Lindros et al. 1980). In sharp contrast to this, a prominent elevation (10 to 110 μM) was found in four of nine recently intoxicated chronic alcoholics. Two others had only slight elevations and, in three alcoholics, changes in blood acetaldehyde after intravenous infusion of ethanol were not significant (Lindros et al. 1980). None of the alcoholics had advanced alcoholic liver disease (cirrhosis). Liver histology was completely normal in three of them. One had a slight, two a moderate, and two a severe fatty degeneration of the liver. One of the alcoholics had alcoholic hepatitis with beginning fibrosis.

Changes in blood acetaldehyde after a high (1.2 g/kg b.wt.) and a low (0.6 g/kg b.wt.) dose of ethanol as related to blood ethanol in one of the alcoholics is shown in figure 1. A marked fluctuation in blood acetaldehyde is clearly demonstrable. After the high as well as the low dose of ethanol, blood ethanol levels exceeded 40 mM during ethanol infusion. Blood acetaldehyde peaks coincided with the blood ethanol peaks. The second dose of ethanol (0.5 or 0.4 g/kg b.wt) again resulted in a simultaneous peak of blood acetaldehyde and ethanol during alcohol infusion. The mean rate of ethanol elimination was significantly higher in nine alcoholics than in three nonalcoholic controls (166 ± 23 v. 107 ± 19 mg/kg b.wt.). The ethanol elimination rate was also higher after the high than the low dose of ethanol, but this difference did not reach a statistical significance (Nuutinen et al. in press).

All infusion experiments were performed on the day immediately after the hospital admission. In one of the alcoholics, the experiment was repeated after 1 week's stay in the hospital. After this period of time without alcohol, the infusion of ethanol did not cause any more of an increase in blood acetaldehyde. This finding indicates that the elevation of blood acetaldehyde during ethanol oxidation most likely is associated with a recent uninterrupted drinking period. Furthermore, the coincidence of blood acetaldehyde and blood ethanol peaks during alcohol infusions suggests an increased rate of ethanol elimination at higher ethanol concentrations. Since the rate of acetaldehyde removal cannot be expected to be inhibited at higher ethanol concentrations, this finding supports the nonuniformity and concentration dependency of blood ethanol elimination (Salaspuro and Lieber 1978).

The Effect of Fructose Infusion

After the early observation of Carpenter and Lee (1937), the stimulatory effect of fructose on ethanol metabolism has been repeatedly confirmed, both in vivo and in vitro (Lundquist 1971). Presumably, fructose stimulates oxidative phosphorylation in order to regenerate ATP, which is consumed by the phosphorylation of fructose. Consequently, the capacity of the respiratory chain to oxidize NADH derived from ethanol is increased (Sholz and Nohl 1976). In our recent study (Lindros et al. 1980), fructose infusion (0.7 g/kg/hr) stimulated the rate of ethanol elimination by 14 percent in nonalcoholic controls and by 17 percent in chronic alcoholics. As demonstrated in figure 1, the enhancement of ethanol elimination by fructose was associated with an elevation of blood acetaldehyde. This effect of fructose could be demonstrated after either the high or the low dose of alcohol in six of the nine chronic alcoholics, but in none of the nonalcoholic controls (Nuutinen et al. in press). The association of the enhanced ethanol elimination induced by fructose with the elevation of blood acetaldehyde levels also supports the role of the rate of ethanol elimination in the regulation of blood acetaldehyde during ethanol oxidation.

The Effect of 4-Methylpyrazole

4-Methylpyrazole is an effective competitive inhibitor of alcohol dehydrogenase (Li and Theorell 1969). It also inhibits many secondary changes in liver metabolism caused by ethanol oxidation (Salaspuro et al. 1977, 1978). In studies with experimental animals, it has been shown to inhibit ethanol elimination by more than 80 percent (Rydberg and Neri 1972), as well as to extinguish blood acetaldehyde completely (Lindros 1975). On this basis, 4-methylpyrazole has recently been recommended by us for the treatment of the disulfiram-alcohol reaction (Lindros et al. in press).

Disulfiram (Antabuse®) and calcium cyanamide (Temposil®, Dipsan®) are widely used in the aversion therapy of alcoholics. Both drugs act primarily by inhibiting aldehyde dehydrogenase (Kitson 1977), thereby causing accumulation of acetaldehyde from ethanol if alcohol is consumed. This results in the disulfiram-alcohol reaction with flushing, tachycardia, headache, palpitations, dyspnea, hyperventilation, hypotension, vertigo, nausea, and vomiting. Sometimes, the reaction may be more severe, with myocardial ischemia or infarction, cardiovascular collapse, respiratory depression, acute congestive heart failure, unconsciousness, and convulsions. At least 20 deaths have been reported (Haley 1979). The established treatment procedures have, for the present, been only supportive and unspecific. As shown in figure 2, the intravenous administration of 4-methylpyrazole to a patient with the disulfiram-alcohol reaction resulted in an immediate normalization of blood acetaldehyde levels, followed by gradual disappearance of associated flushing, tachycardia, and substernal pains secondary to an ischemic heart attack (Lindros et al. in press). In subsequent experiments with human volunteers, 4-methylpyrazole was shown to be effective also (Lindros et al. in press) in the management of the reaction produced by the simultaneous intake of calcium carbimide and ethanol.

Acetaldehyde Removal and Blood Acetaldehyde

As mentioned previously, drugs such as disulfiram and calcium cyanamide, which inhibit aldehyde dehydrogenase, are the best known and most common causes of increased blood acetaldehyde. There also exist, however, other aldehyde dehydrogenase inhibitors; for instance, coprine of inky mushroom and sulfonylureas, which may induce flushing and other symptoms of the disulfiram-alcohol reaction when used in combination with alcohol (Lindros 1973). In addition to these agents, however, the activity of hepatic aldehyde dehydrogenase also may be decreased because of either acquired (chronic alcoholics) or hereditary reasons (Orientals).

In Chronic Alcoholics

Most of the acetaldehyde produced during ethanol oxidation is oxidized by the liver (Lindros 1973). In rats, mitochondrial low-K_m acetaldehyde dehydrogenase is the most important determinant in the regulation of the hepatic acetaldehyde oxidation during ethanol metabolism (Tottmar and Marchner 1975). Chronic ethanol consumption results in a significant reduction of the capacity of rat liver mitochondria to oxidize acetaldehyde, probably due to the decreased ability to reoxidize NADH (Hasumura et al. 1975). Moreover, chronic heavy feeding of alcohol to rats also may decrease the activity of hepatic low-K_m aldehyde dehydrogenase (Koivula and Lindros 1975).

Jenkins and Peters (1980) found that aldehyde dehydrogenase activity in liver biopsy specimens of 12 noncirrhotic alcoholics was considerably lower than in 11 control subjects. In addition, their subcellular fraction studies indicated a selective reduction of the cytosolic component of acetaldehyde dehydrogenase. In preliminary studies with chronic alcoholics, we found that the activities of both the cytosolic high K_m and the mitochondrial low K_m aldehyde dehydrogenase are significantly decreased ($p<0.001$) in chronic alcoholics as compared to nonalcoholic controls (table 1). None of these alcoholics had advanced alcoholic liver injury. The activities of aldehyde dehydrogenase were analyzed fluorimetrically from liver biopsy specimens obtained at laparoscopy (Nuutinen et al. in press). Two different acetaldehyde concentrations (18mM and 180 μM) were used in order to differentiate the high and low K_m enzymes. In addition to the decreased hepatic activity of the enzyme, we also found a significant negative correlation ($r= -0.830$) between the peak blood acetaldehyde and the activity of mitochondrial low K_m aldehyde dehydrogenase (Nuutinen et al. in press). These findings suggest that the main reason for blood acetaldehyde elevation in chronic alcoholics is the decreased activity of hepatic low K_m aldehyde metabolizing enzyme.

Racial Differences

Biological and behavioral differences in response to alcohol intake have been observed among various racial and ethnic groups (Wolff 1972; Zeiner et al. 1979). The alcohol-induced sensitivity in Orientals, for instance, includes several symptoms and signs common to the alcohol-disulfiram reaction: facial flushing, increased skin temperature, peripheral vasodilation, tachycardia, nausea, and abdominal discomfort. In fact, higher blood acetaldehyde levels have been noted during ethanol oxidation in Japanese and Chinese subjects who show facial flushing than in nonflushing subjects (Ijiri 1974; Zeiner et al. 1979). Originally, this was attributed to the highly active atypical alcohol dehydrogenase that occurs in about 85 percent of Japanese subjects (Stomatoyannopoulos et al. 1975). However, no significant difference in the rate of alcohol metabolism has been found in normal and atypical ADH phenotype carriers (Edwards and Price-Evans 1967).

Most recently, electrophoretic and kinetic studies on autopsy liver specimens from individuals of different racial groups have revealed a polymorphism in aldehyde dehydrogenase (Agarwal et al. 1981). About 52 percent of the Japanese livers have unusual aldehyde dehydrogenase, whereas none was found in liver specimens of German subjects. Using hair root as a source of aldehyde dehydrogenase, it was also demonstrated that the unusual phenotype of the enzyme was associated with flushing. These findings suggest that the alcohol sensitivity of Orientals is caused by delayed oxidation of acetaldehyde by an unusual acetaldehyde dehydrogenase.

Conclusions

In man, blood levels of acetaldehyde reflect a subtle balance between the rate of its formation from ethanol and its further oxidation in the liver. A change either in formation or in oxidation is likely to induce changes, especially if both processes are acting in the same direction. This was clearly demonstrated in experiments in which fructose was used to stimulate ethanol metabolism in chronic alcoholics with reduced activities of hepatic aldehyde dehydrogenase. The conclusions in this paper, however, are drawn from studies in which acetaldehyde has been determined from the peripheral venous blood. It should be borne in mind that these values are influenced by the dilution in the inferior caval vein and oxidation in peripheral tissues. In fact, our preliminary studies suggest that acetaldehyde concentration is considerably higher in the hepatic than in the peripheral vein. Therefore, the acetaldehyde level in the liver during alcohol oxidation is also most probably several times higher than in other tissues. On this basis, the detrimental effects of alcohol (Lieber et al. 1980) are likely to be concentrated in this organ.

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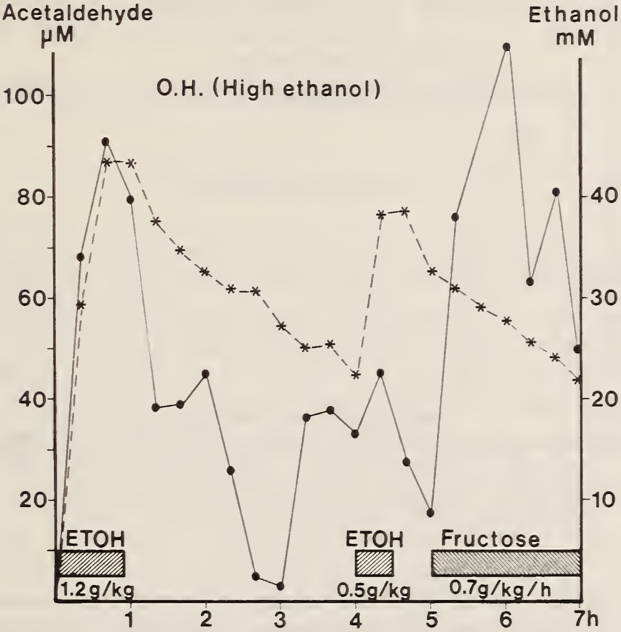
Figure 1. Blood Acetaldehyde (•————•) and Ethanol (*----*) During and After an Intravenous Infusion of a High and a Low Dose of Ethanol and During Fructose Stimulation in a Chronic Alcoholic



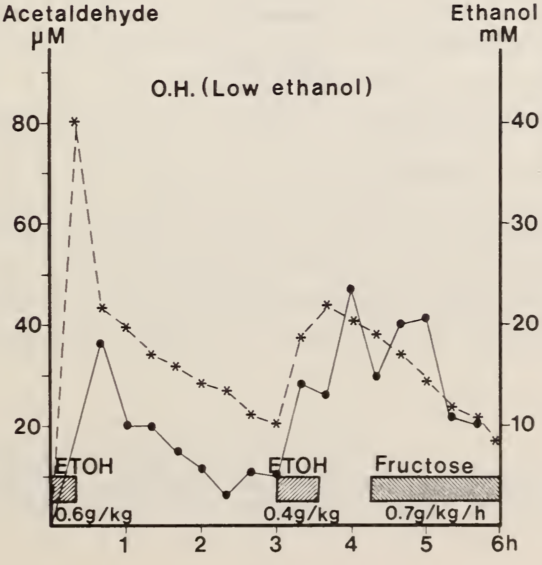


Figure 2. The Effect of 4-Methylpyrazole on Blood Acetaldehyde in a Chronic Alcoholic with the Disulfiram-Alcohol Reaction

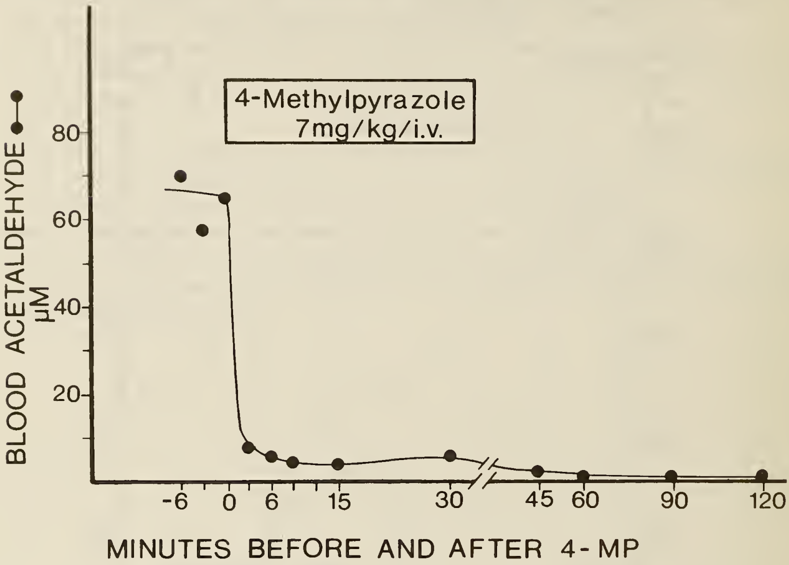


Table 1. Activities of Hepatic Aldehyde Dehydrogenase in Chronic Alcoholics and Nonalcoholic Controls (mU/mg Protein)

	Controls (4)	Alcoholics (6)
High K_m (18mM)	26.0 ± 14.2	7.7 ± 4.7
Low K_m (180μM)	12.9 ± 8.8	3.0 ± 1.0

Mean ± S.D.

Cyanamide Differentiates Between Direct and Indirect Effects of Acetaldehyde on Gluconeogenesis and the Oxidation of Pyruvate*

Elisa Dicker and Arthur I. Cederbaum

Introduction

The oxidation of pyruvate by mitochondria isolated from rat liver, kidney, muscle, and brain was found to be especially sensitive to inhibition by acetaldehyde (Cederbaum and Rubin 1977). Concentrations of acetaldehyde which inhibited the oxidation of pyruvate had no effect on the oxidation of glutamate, β-hydroxybutyrate α-ketoglutarate, succinate, or ascorbate (Cederbaum et al. 1974, 1976). The mechanism responsible for this unique sensitivity to acetaldehyde was not established. Two mechanisms that could be responsible for this sensitivity to acetaldehyde can be proposed: (1) A direct effect of acetaldehyde on pyruvate dehydrogenase due either to active complexation of acetaldehyde with the thiamine pyrophosphate cofactor of the pyruvate dehydrogenase complex or to reaction of acetaldehyde with essential sulfhydryl groups of the enzyme (Blass and Lewis 1973) or (2) an indirect metabolic effect of acetaldehyde; that is, a mechanism that requires the oxidation of acetaldehyde with subsequent changes in the mitochondrial $NAD^+/NADH$, ADP/ATP, and acetyl CoA/CoA ratios. Changes in these ratios are known to regulate the activity of pyruvate dehydrogenase (Randle et al. 1978).

Recent experiments by our laboratory studied the effects of acetaldehyde on gluconeogenesis from various precursors by isolated liver cells in order to evaluate the role of acetaldehyde in the actions of ethanol (Cederbaum and Dicker 1979, 1980). In the presence of pyrazole, acetaldehyde inhibited glucose production from xylitol, sorbitol, and glycerol, whereas glucose production from pyruvate was increased. It was not determined whether these effects of acetaldehyde on gluconeogenesis required the metabolism of acetaldehyde, with

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NOTE: Figures and tables appear at end of paper.

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subsequent changes of the cellular NAD+/NADH redox ratio, or reflected direct effects of acetaldehyde.

Cyanamide is an effective inhibitor of aldehyde dehydrogenase (Marchner and Tottmar 1976*a,b*) and prevents the metabolism of acetaldehyde. Hence, cyanamide may be a useful tool to attempt to distinguish between direct and indirect metabolic effects of acetaldehyde on cellular metabolism. In the current report, the ability of cyanamide to block the oxidation of acetaldehyde by isolated mitochondria and liver cells and to prevent the effects of acetaldehyde on pyruvate oxidation and on gluconeogenesis was evaluated. Full details and methods are reported elsewhere (Cederbaum 1981; Dicker and Cederbaum in press).

Results and Discussion

Effects of Cyanamide on the Mitochondrial Oxidation of Acetaldehyde and on the Activity of Aldehyde Dehydrogenase

Isolated rat liver mitochondria, in state 4, oxidized acetaldehyde at a rate of 9.7 nmol/min/mg protein (table 1). This rate of oxidation is inhibited by 90 percent by rotenone, indicating that the oxidation of acetaldehyde is NAD+-dependent. The addition of ADP (state 3) increases acetaldehyde oxidation by 30 percent (table 1). Cyanamide (0.003-0.33 mM) after a 3-min incubation period with the mitochondria, inhibited the state 4 (62 to 86 percent), as well as the state 3 (19 to 93 percent), rate of oxidation of acetaldehyde (table 1). Without this incubation period, cyanamide did not affect acetaldehyde oxidation. The ability of cyanamide to inhibit mitochondrial oxidation of acetaldehyde was confirmed by assaying the activity of aldehyde dehydrogenase in deoxycholate-disrupted mitochondria. The activity of aldehyde dehydrogenase measured in the presence of 0.2 mM acetaldehyde as substrate (19 nmol/min/mg protein) was similar to the state 3 rate of oxidation of acetaldehyde by intact mitochondria, indicating that the uptake of acetaldehyde in state 3 is regulated by the activity of aldehyde dehydrogenase. Cyanamide (0.003 to 0.330 mM), after a 3-min incubation period, inhibited the activity of aldehyde dehydrogenase by 67 to 74 percent. Cyanamide had no effect on the state 4 or state 3 rate of oxidation of other mitochondrial substrates tested (e.g., succinate or glutamate), demonstrating that cyanamide is an effective specific inhibitor of acetaldehyde oxidation by isolated mitochondria.

Effect of Cyanamide on the Inhibition of Pyruvate Oxidation by Acetaldehyde

Acetaldehyde at concentrations of 0.2 to 0.3mM inhibited the state 3 rate of pyruvate oxidation (table 2). These levels of acetaldehyde did not affect the oxidation of glutamate or succinate, pointing to the unique sensitivity of pyruvate oxidation to acetaldehyde. There was no effect of acetaldehyde on the state 4 rate of pyruvate oxidation. In state 4, the rate of oxidation of pyruvate is regulated by the reoxidation of NADH; whereas, in state 3, the rate of pyruvate oxidation is regulated by the activity of pyruvate dehydrogenase.

To determine whether this unique sensitivity to acetaldehyde represents a direct toxic effect of acetaldehyde, or requires the metabolism of acetaldehyde, experiments with cyanamide were carried out. Cyanamide itself did not affect the rate of pyruvate oxidation. The addition of 0.1 mM cyanamide did not prevent the inhibition of pyruvate oxidation by acetaldehyde (table 2), although the oxidation of acetaldehyde was inhibited greater than 90 percent. These results suggest that the inhibition by acetaldehyde reflects a direct, rather than a metabolic, effect of acetaldehyde on pyruvate dehydrogenase. Acetaldehyde is known to react rapidly with sulfhydryl groups, and pyruvate dehydrogenase contains active sulfhydryl groups which are required for activity. Hence, the direct toxic action of acetaldehyde may reflect interaction with essential sulfhydryl groups of the complex.

Effect of Cyanamide on Acetaldehyde Oxidation by Isolated Rat Liver Cells

In the presence of pyrazole, to prevent reduction of acetaldehyde to ethanol via the activity of alcohol dehydrogenase, liver cells oxidized acetaldehyde (0.2 to 0.67 mM) at rates of 7.5 to 10 nmol/min/mg liver cell protein (figure 1). Cyanamide, at concentrations of 0.01 to 0.1mM, inhibited the oxidation of acetaldehyde by 70 to 90 percent (figure 1). These results demonstrate that cyanamide can be used as an effective in vitro inhibitor of acetaldehyde oxidation by isolated intact rat liver cells. Cyanamide had no effect on hepatic oxygen consumption.

Effect of Cyanamide and Acetaldehyde on Glucose Production From Xylitol, Sorbitol, Pyruvate, and Glycerol

Acetaldehyde, in the absence of pyrazole, stimulated glucose production from xylitol and sorbitol (figure 2). The stimulation was suggested to reflect the reduction of acetaldehyde to ethanol via alcohol dehydrogenase with the subsequent regeneration of NAD$^+$ (Cederbaum and Dicker 1980). The availability of NAD$^+$ regulates glucose production from xylitol and sorbitol (Berry et al. 1973; Williamson et al. 1971). If this mechanism is correct, then cyanamide, which does not affect alcohol dehydrogenase, should not prevent the stimulatory effects of acetaldehyde. Indeed, cyanamide (0.1 mM) did not prevent the stimulatory action of acetaldehyde on glucose production from xylitol and sorbitol (figure 2). This concentration of cyanamide inhibited the oxidation of acetaldehyde by 80 percent. These results indicate that the stimulatory effect of acetaldehyde is mediated via alcohol dehydrogenase and not aldehyde dehydrogenase.

In the presence of pyrazole, acetaldehyde inhibited glucose production from xylitol, sorbitol, and glycerol, whereas glucose production from pyruvate was stimulated (figures 2 and 3). Cyanamide (0.1 mM) prevented the inhibitory as well as the stimulatory actions of acetaldehyde on glucose production from these substrates (figures 2 and 3). These results indicate that the actions of acetaldehyde on glucose production do not reflect a direct toxic effect of acetaldehyde but rather require the active metabolism of acetaldehyde.

Since acetaldehyde metabolism is NAD$^+$-dependent, acetaldehyde blocks glucose production from NAD$^+$-dependent precursors such as xylitol, sorbitol, and glycerol (figures 2 and 3). Cyanamide, by blocking acetaldehyde oxidation, prevents the inhibitory effect of acetaldehyde on glucose production from those substrates. Acetaldehyde stimulates glucose production from pyruvate, probably by providing NADH for the triose phosphate dehydrogenase reaction (Cederbaum and Dicker 1980). This stimulation is also prevented by cyanamide, which blocks the oxidation of acetaldehyde. Consistent with this mechanism is the observation that acetaldehyde elevates the β-hydroxybutyrate/acetoacetate ratio (a measure of the mitochondrial NADH/NAD$^+$ redox ratio), and this increase was totally reversed by cyanamide (Dicker and Cederbaum in press).

Conclusions

In order to differentiate between direct toxic actions and indirect metabolic actions of acetaldehyde on cellular metabolism, experiments with cyanamide were carried out.

1. Cyanamide, after a brief incubation period, was found to block the oxidation of acetaldehyde by isolated mitochondria and by intact isolated rat liver cells. Therefore, cyanamide is a useful tool to inhibit acetaldehyde oxidation in in vitro systems.

2. Pyruvate oxidation by mitochondria is especially sensitive to inhibition by acetaldehyde. This inhibition was not prevented by cyanamide, indicating that acetaldehyde has a direct toxic effect on the primary enzyme, pyruvate dehydrogenase.

3. The actions of acetaldehyde on glucose production from xylitol, sorbitol, glycerol, and pyruvate were prevented by cyanamide, indicating that these actions reflect a metabolic effect of acetaldehyde and not a direct toxic effect.

4. These results demonstrate that, depending on the reaction being studied, acetaldehyde may have direct toxic effects as well as metabolic indirect effects on cellular metabolism.

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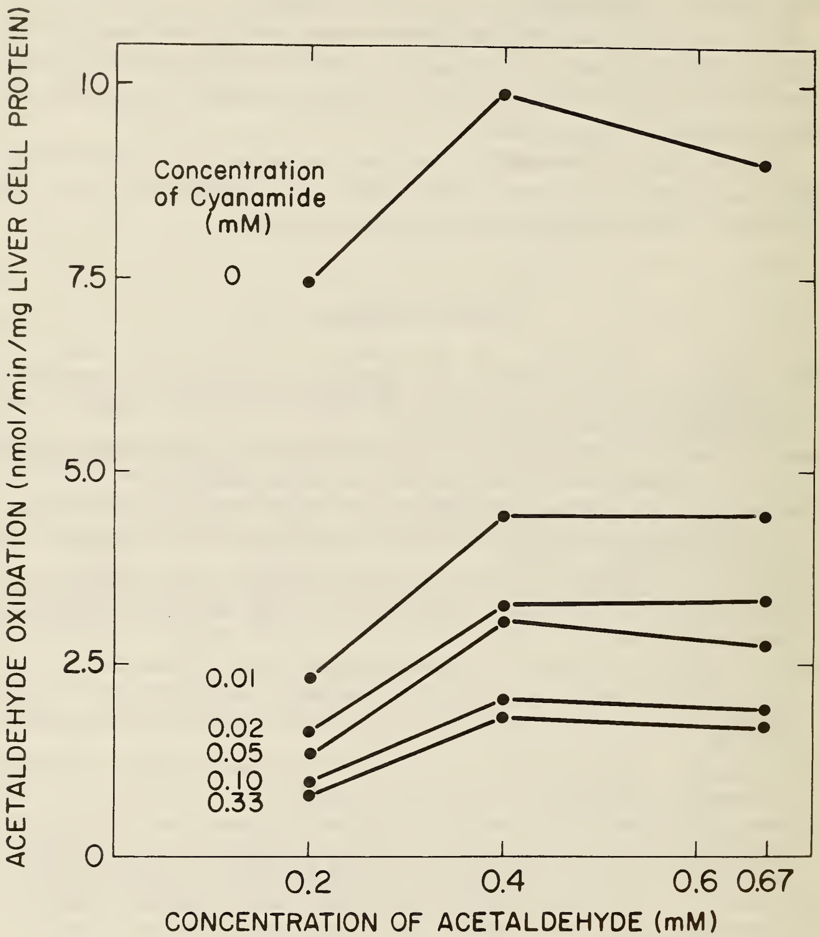
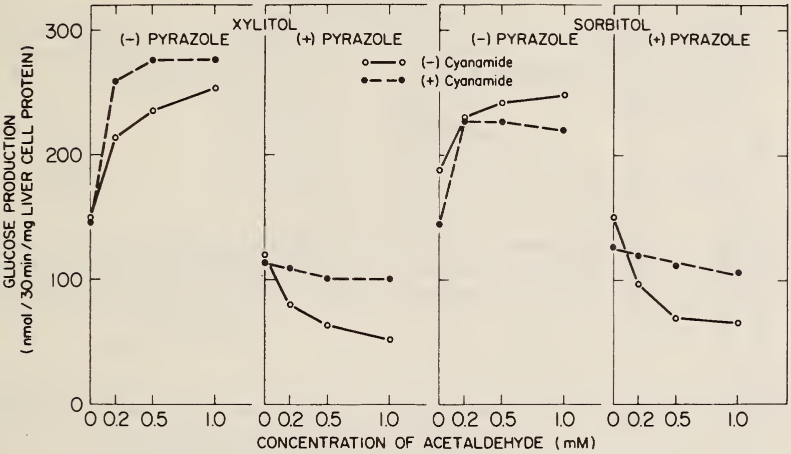
Figure 1. Effect of Cyanamide on the Oxidation of Acetaldehyde by Isolated Rat Liver Cells



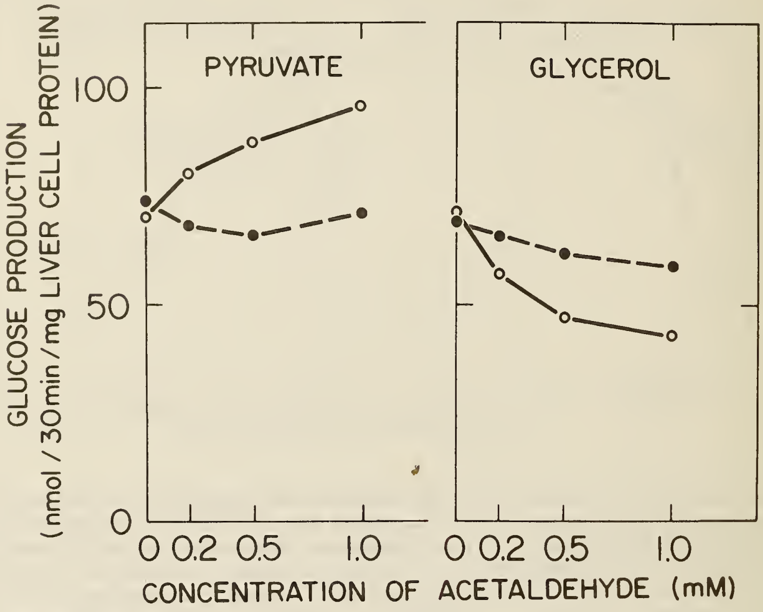
Liver cells were incubated for 5 min with cyanamide before initiating the reaction by addition of acetaldehyde. The reaction was carried out in the presence of 3 mM pyrazole to prevent reduction of acetaldehyde back to ethanol. Results are the means of three experiments.

Figure 2. Effect of Acetaldehyde on Glucose Production From Xylitol and Sorbitol in the Absence or Presence of Cyanamide



Liver cells were incubated with 0.1 mM cyanamide for 5 min before starting the reaction by addition of either xylitol or sorbitol and the indicated concentration of acetaldehyde. When present, the final concentration of pyrazole was 3 mM. Results are the means of four experiments. In the absence of pyrazole, acetaldehyde stimulated glucose production and this stimulation *was not* prevented by cyanamide. In the presence of pyrazole, acetaldehyde inhibited glucose production and this inhibition *was* prevented by cyanamide.

Figure 3. Effect of Acetaldehyde on Glucose Production From Pyruvate and Glycerol in the Absence or Presence of Cyanamide



Conditions were as in figure 2. Pyrazole was present at 3 mM. Results are from four experiments.

○——○	− cyanamide
●----●	+ cyanamide

Table 1. Effect of Cyanamide on the Oxidation of Acetaldehyde and on the Activity of Aldehyde Dehydrogenase

Concentration of Cyanamide (mM)	Acetaldehyde oxidation (nmol/min/mg protein) State 4	Acetaldehyde oxidation (nmol/min/mg protein) State 3	Activity of Aldehyde Dehydrogenase (nmol/min/mg protein)
0	9.69 ± 1.34	12.69 ± 1.66	18.9 ± 0.37
0.003	3.70 ± 1.11	10.27 ± 1.37	
0.033	1.38 ± 0.76	0.91 ± 0.57	6.2 ± 0.66
0.330	1.37 ± 0.83	0.72 ± 0.43	4.9 ± 0.49

Mitochondria were incubated with cyanamide or water 3 min before initiating the reaction with 0.2 mM acetaldehyde. Results are from four experiments. States 4 and 3 refer to experiments carried out in the absence or presence of 3.3 mM ADP, respectively.

Table 2. Effect of Acetaldehyde, Cyanamide or Acetaldehyde Plus Cyanamide on Pyruvate Oxidation

Concentration of Acetaldehyde (mM)	Cyanamide (0.10 mM)	Pyruvate oxidation (natom oxygen/min/mg protein)	Effect of Acetaldehyde (percent)
0	−	60.69 ± 3.81	
0	+	58.57 ± 4.23	
0.2	−	51.89 ± 3.73	− 15
0.2	+	48.55 ± 2.94	− 20
0.3	−	48.94 ± 2.47	− 19*
0.3	+	43.28 ± 2.42	− 29**
0.5	−	45.39 ± 2.47	− 25*
0.5	+	42.86 ± 1.70	− 29**
1.0	−	42.90 ± 2.72	− 29**
1.0	+	38.50 ± 2.44	− 37**
2.0	−	40.67 ± 1.00	− 33**
2.0	+	36.41 ± 0.43	− 40**

Mitochondria were incubated with cyanamide or water for 3 min before initiating the reaction with acetaldehyde. Pyruvate (10 mM) plus malate (3 mM) was added and oxygen uptake was recorded. Cyanamide did not prevent the inhibitory action of acetaldehyde on pyruvate oxidation, indicating that acetaldehyde has a direct toxic effect on pyruvate dehydrogenase.

* $p<0.01$

** $p<0.005$

Acetaldehyde Mediated Disruption of Liver Microtubules After Alcohol Consumption*

Enrique Baraona, Yoshiro Matsuda, Pekka Pikkarainen, Frances Finkelman, and Charles S. Lieber

Abstract

Alcohol decreases liver microtubules. This effect is attenuated by inhibiting ethanol oxidation with 4-methylpyrazole and accentuated by decreasing acetaldehyde oxidation with disulfiram. Chronic alcohol consumption exaggerates the effect of ethanol on microtubules, increases ethanol oxidation, and acetaldehyde accumulation in the liver. Acetaldehyde binds to tubulin, competes with colchicine binding and inhibits tubulin polymerization.

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* Full article to be published: Baraona, E.; Matsuda, Y.; Pikkarainen, P.; Finkelman, F.; and Lieber, C.S. Effect of ethanol on hepatic protein secretion and microtubules. Possible mediation by acetaldehyde. In: *Currents in Alcoholism*, Galanter, M., ed. Grune & Stratton, New York, Vol. VIII. (in press).

The Significance of the Increased Ethanol Oxidation by the Microsomal Ethanol Oxidizing System (MEOS) and the Mitochondrial Function for the Elevation of Acetaldehyde: A Major Cause of Alcoholic Visceropathy*

Shohei Matsuzaki and Charles S. Lieber

Abstract

It has been assumed that the toxicity of acetaldehyde produced during ethanol oxidation may play an important role to cause alcoholic visceropathies in alcoholics. In recent studies the toxic effects of acetaldehyde at its low levels, which can occur in vivo, have been clarified experimentally. The blood concentration of acetaldehyde, however, has still been disputed, including the problems of its nonenzymatic production and binding to proteins. After the careful evaluation of these modifying factors of acetaldehyde levels in the blood and tissues, the almost real value of blood acetaldehyde was found to be apparently measurable under the certain analytical condition.

The high level of blood acetaldehyde, which was making a plateau, was found at high blood ethanol levels in our human study. Furthermore, the level was higher in alcoholics than in nonalcoholic subjects only at high ethanol levels. The high level at high ethanol concentrations and the difference between the two groups strongly suggested the participation of the microsomal ethanol oxidizing system (MEOS) to produce acetaldehyde and the effect of decreased disposition of it by impaired mitochondria in the liver after chronic ethanol consumption.

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* Full articles published in: Matsuzaki, S.; Gordon, E.; and Lieber, C.S. Increased ADH independent ethanol oxidation at high ethanol concentrations in isolated rat hepatocytes: The effect of chronic ethanol feeding. *J Pharmacol Exp Ther*, 217:133-137, 1981; and Matsuzaki, S., and Lieber, C.S. Increased susceptibility of hepatic mitochondria to the toxicity of acetaldehyde after chronic ethanol consumption. *Biochem Biophys Res Commun*, 75:1059-1065, 1977.

The effect of ethanol concentrations on ethanol oxidation rates and acetaldehyde levels were experimentally studied in conjunction with acetaldehyde oxidizing activity using isolated hepatocytes of rats. The results revealed that acetaldehyde was elevated strikingly at high ethanol levels, only when ethanol oxidation rates by the cells exceeded that of acetaldehyde. The level reached nearly a plateau when it became higher than about 300 μM in the incubation medium.

Effects of Ethanol on Protein Synthesis and Secretion*

Marcus A. Rothschild, Murray Oratz, Jorg Mørland, Sidney S. Schreiber, Alvin Burts, and Barbara Martin

Abstract

This paper discusses the complexities of understanding protein synthesis in an *in vivo* model, with citations of a number of studies, and advises that extrapolation of data obtained from one model system to another must be done with caution. The authors discuss findings that in patients with cirrhosis of the liver secondary to chronic ethanol consumption, serum albumin levels are depressed, and they discuss serum albumin synthesis. The relationship between ethanol consumption and albumin synthesis and secretion is discussed, based on a number of research studies on animal livers. The authors conclude that while ethanol ingestion or metabolism interferes with protein synthesis, it is not known if an intermediate metabolic step (or steps) is involved. Preliminary studies suggest that the nature of protein synthesis is complex, even in isolated systems, and further studies are needed, they say.

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* Full article published in: *Pharm Biochem Behav*, 12(1):31-36, 1980.

Binding of Acetaldehyde to Rat Liver Cellular Protein*

Fumio Nomura and Charles S. Lieber

Abstract

The authors report on experiments using rats, designed to determine if acetaldehyde could play a role in the binding of reactive metabolites to liver proteins; it has been suggested that this binding may be important in the production of liver injury. Their results showed that: exogenously added acetaldehyde binds to rat hepatic mitochondria and microsomes; that acetaldehyde formed endogenously from ethanol by the microsomal ethanol oxidizing system (MEOS) also binds to rat liver microsomes and its binding is greater than that of similar concentrations of exogenous acetaldehyde; that after chronic alcohol consumption, there is an increase in the binding of acetaldehyde generated from ethanol by MEOS, in association with the enhanced MEOS activity; and that thiol groups and free amino groups appear to be involved in the binding of acetaldehyde. The authors suggest that part of the toxicity of ethanol may be mediated by the binding of its reactive metabolite, acetaldehyde, to liver protein, and call for further research into the specific effects of the binding.

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* Full article published in: Nomura, F., and Lieber, C.S. Binding of acetaldehyde to rat liver microsomes: Enhancement after chronic alcohol consumption. *Biochem Biophys Res Commun*, 100:131-137, 1981.

SESSION VI: LIPIDS AND BILE ACIDS

Ethanol and Biomembranes

Ellen R. Gordon

A vast amount of data has been collected on the in vitro direct effects of ethanol on the functional capacity of biomembranes. Many of these studies have been conducted in the presence of concentrations of ethanol much higher than those encountered in vivo. The significance of such data is difficult to evaluate and will not be reviewed here. However, a survey of the literature does indicate that membrane-associated functions are altered notably in the nervous system and in the liver at physiological levels of ethanol in animals chronically fed ethanol or after an acute dose of ethanol. These changes are numerous and, at first glance, do not seem to have a common etiology.

It has long been postulated that the molecular site of the action of ethanol is direct and at the level of the membrane. This is based on the observation of Meyers and Overton (1926) that the potency of a series of alcohols in affecting a variety of biological processes is roughly proportional to their lipid-water partition coefficient, suggesting that their penetration into the lipid regions of the membrane results in their effect. Thus, the insertion of the alcohol into the membrane may directly alter the physical properties of the membrane.

Recent studies by Lyon and colleagues (1980) tend to support this theory. Utilizing a sensitive electron paramagnetic resonance technique, they examined the membrane-disordering ability of a series of alcohols. Each alcohol was capable of a concentration-dependent disordering of brain synaptosomal plasma membranes isolated from Swiss Webster mice. A linear correlation was found between the membrane-disordering potency for each alcohol and its oil/water partition coefficient. This suggests that the extent of membrane disordering by each alcohol is directly related to its relative lipid solubility. Earlier studies by this group (Chin et al. 1979) demonstrated that the physical state of mouse biomembranes was changed in the presence of a low concentration of ethanol. The addition of ethanol (0.02 to 0.04 M) increased the fluidity of the erythrocytes and synaptosomal membranes, but had no effect on myelin. These acute in vitro experiments were repeated, utilizing membranes from animals chronically treated with ethanol. In these experiments, the synaptosomal and erythrocyte membranes were more resistant to the in vitro fluidizing effects of ethanol than were membranes prepared from their respective pair-fed controls. Another group (Johnson et al. 1979) has found similar results. These data appear to support a theory proposed by Hill and Bangham (1975) that mammalian cells can adapt to drugs by changing the lipid composition of the membrane. In fact, Chin, Goldstein, and Parson (1979) were able to demonstrate that the membranes from the ethanol-fed mice contained a higher concentration of cholesterol than did their respective controls. However, the small increase in cholesterol did not alter the microviscosity of the membranes unless ethanol was added in vitro. It appears that the increase in cholesterol may have simply decreased the partition of ethanol in the membrane, as has been demonstrated with barbiturates (Miller and Yu 1977).

Similar studies have been conducted by others (Grieve et al. 1979). It has been shown that Swiss Webster mice (which had received a diet supplemented with saturated fat) required a higher blood ethanol concentration to produce loss of righting reflex than did mice receiving the control diets. In addition, the administration of the inhibitor of cholesterol synthesis, diazocholesterol, throughout life prevented this protective effect. These results appear to be compatible with the hypothesis that the tolerance to ethanol in mice may be associated with a change in the physical state of the membranes of the brain, produced by changes in the fatty acid composition of the phospholipids and the cholesterol content of the membrane (Littleton and John, 1977; Littleton et al. 1979; Westcott and Weiner 1980).

That the acute and chronic consumption of ethanol produces a multiplicity of changes in the functions of the membranes of the hepatocytes has been well documented (Lieber 1980). The etiology of these changes, however, has not been clarified. Chronic ethanol consumption causes profound changes in the ultrastructure of hepatic organelles, particularly mitochondria, in rats, primates, and man (Lieber 1977). Hepatic electron micrographs indicate that many mitochondria are enlarged and have bizarre shapes. These morphologic changes are associated with alterations in the metabolic functions of the mitochondrial membranes. Isolated liver mitochondria from rats chronically fed ethanol exhibit impaired respiration, detected mainly as a decrease in respiratory rates linked to the oxidation of NAD^+-dependent substrates and succinate (Bernstein and Pennialli 1978; Cederbaum et al. 1974; Gordon 1973; Spach et al. 1979). Phosphorylation capacity of the mitochondria, as indicated by ADP/O ratio, has also been reported to be decreased (Cederbaum et al. 1974; Gordon 1973) or unchanged (Spach et al. 1979).

Similar changes have been observed in submitochondrial particle prepared from ethanol-fed rats (Thayer and Rubin 1979). It has been concluded that these effects may be a consequence of the decreased content of cytochrome oxidase and impaired functional capacity of mitochondrial ATPase. In this respect, it is of interest that both these enzymes are membrane bound, and their activities are influenced by their lipid environment (Razin 1972; Sandermann 1978). Thus, these changes in mitochondrial function may indeed be explained by ethanol-produced alterations in the physical state of the membrane.

Recent studies by Hosein and his colleagues (1977) appear to support this hypothesis. They assessed the integrity of hepatic mitochondrial membranes by measuring the activity of Mg^{++}-stimulated ATPase at different temperatures. Arrhenius plots of the data demonstrated a distinct change in activity at 32°C in mitochondrial preparations from ethanol-fed rats, but a similar change was not detected in the preparations from control animals. These data were obtained from mitochondrial preparations from ethanol-fed rats in the absence of ethanol, suggesting that the consumption of ethanol had altered the composition of the membrane.

Investigations from several laboratories indicate that changes observed in the fatty acid composition of hepatic mitochondria in ethanol-fed animals depended on the lipid composition of the diet, the duration and amount of ethanol consumed, and the sex of the animal (Comporti et al. 1971; French et al. 1971; Thompson and Reitz 1976, 1978). A decrease in arachidonic acid and C22 polyenes has been noted in male rats maintained on diets either low or high in fat content with ethanol isocalorically replacing the carbohydrate. This change was associated with an increase in $18{:}2\omega6$, suggesting a decreased desaturase activity similar to that seen following a period of short starvation (Pugh and Kates 1977). Mitochondrial preparations from female rats treated in a similar manner did not exhibit this change in polyunsaturated fatty acids.

That ethanol consumption produces changes in the functions of hepatic microsomes has been well established. Numerous studies have been concerned with the interaction between ethanol and the metabolism of drugs and other foreign compounds, both in vivo and in vitro (Lieber and Pirola in press). The most often observed effects have been inhibitory and ascribed to a direct inhibitory action of ethanol on the microsomal mixed function oxidase pathway. The nature of this inhibitory action is not yet fully established, but is generally believed to be due to an interference of ethanol with the binding of the substrate or displacement of bound substrate by ethanol. These are general effects, seen in the presence of ethanol. However, it has also been demonstrated that the chronic consumption of ethanol produces not only a proliferation of the smooth endoplasmic reticulum, but also a change in the specific activity of many of the membrane-bound enzymes (such as the mixed function oxygenase), the microsomal ethanol oxidizing system, and NADPH cytochrome c P-450 reductase (Joly et al. 1976, 1977; Ohnishi and

Lieber 1977; Teschke et al. 1972, 1975). Moreover, it has also been shown that microsomal cytochrome P-450, a reductase, and phospholipids play a key role in the microsomal hydroxylation of various drugs (Lu et al. 1969). Whether these changes in the enzyme activity are the result of alteration in the microenvironment in the membrane has not been established. If ethanol oxidation changes the physical state of the mitochondrial membranes by alterations in lipid or calcium content, then similar changes could also be expected in the microsomal membrane. The effects of chronic intake of ethanol on the chemical components of the microsomal membranes vary depending on the species, the diet, and the duration of ethanol intake (Comporti et al. 1971; Gordon et al. 1981; Joly et al. 1977). The administration of an acute dose of ethanol to starved ethanol-fed rats produces a significant reduction in the polyunsaturated fatty acid content of their hepatic microsomal phospholipids (Shaw et al., 1981). Such a change would be expected to markedly alter the physical state of the membrane. However, further studies are required to ascertain if the numerous ethanol-induced changes in microsomal function are related to rule out changes in the physical state of the membranes.

Thus, ethanol at physiological levels can affect the functions of biomembranes in two distinct ways. As a result of ethanol oxidation, lipid metabolism is altered, producing changes in the phospholipid and cholesterol content of the membrane, two important determinants of the physical state of the membrane. This, in turn, could alter the function state of the membrane, which would persist following the disposition of ethanol. Ethanol also exerts a pharmacologic effect because of its lipid solubility, and its potency is determined by the nature of the lipid composition of the membrane.

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Lipid and Lipoprotein Abnormalities in Alcoholic Liver Disease*

Seymour M. Sabesin

Role of the Liver in Lipoprotein Metabolism

The derangements in blood lipids and lipoproteins that occur in association with alcohol-induced liver injury reflect profound abnormalities in the biosynthesis and metabolism of lipids and lipoproteins. Since the liver is essential for many aspects of lipid and lipoprotein synthesis and metabolism, it is likely that the striking derangements in the concentration and composition of lipoproteins found in patients with alcoholic hepatitis reflect an impairment of one or more of these processes. In order to appreciate the importance of the liver in this regard, and the profound abnormalities that occur in alcoholic hepatitis, it is valuable to discuss briefly certain fundamental aspects of lipoprotein metabolism (Eisenberg and Levy 1975; Morrisett et al. 1975).

The main function of lipoproteins is the transport of lipids in the blood. Except for the intestinal formation of chylomicrons after a fat-containing meal, the liver is the major source of plasma lipoproteins. In addition to its role in lipoprotein formation, the liver subserves many other essential functions in lipoprotein metabolism (Sabesin 1979; Sabesin et al. 1979, 1980). The liver synthesizes so-called very low density lipoproteins (VLDL) and a precursor of high density lipoproteins (HDL) called nascent HDL. The liver synthesizes several key enzymes in lipoprotein metabolism and also many of the proteins (apoproteins) that are constituents of the lipoprotein particles. Some of these apoproteins are also important for lipoprotein metabolism, since they serve as activators of enzymes—such as lipoprotein lipase and lecithin: cholesterol acyltransferase—essential in the metabolic pathways. The liver is also involved in the removal from the blood of the end products ("remnants") of plasma lipoprotein catabolism and in remnant degradation. Finally, the liver helps to regulate total body cholesterol sources by secreting cholesterol into the bile.

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Characteristics of Plasma Lipoproteins

The major classes of plasma lipids—triglycerides, cholesterol, cholesteryl esters, and phospholipids—combine in varying proportions with specific apoproteins to form the lipoprotein molecules. The primary function of the lipoproteins is the transport of lipids in the watery environment of the blood. The structure of the lipoprotein molecule permits hydrophobic lipids (triglycerides, cholesteryl esters) to form the core of a particle which is covered by a surface layer of phospholipids and apoproteins.

Lipoprotein formation occurs primarily in hepatocytes (VLDL and HDL) and also within intestinal mucosa epithelial cells in response to dietary lipid absorption (chylomicrons). Within cells the lipids combine with specific apoproteins to form the lipoproteins which are secreted into the plasma or lymph where they undergo rapid metabolic transformations involving triglyceride removal, cholesterol esterification, and exchange of lipids and apoproteins. These metabolic transformations result in the formation of lipoprotein particles, which are characteristic in size and composition to the lipoproteins which can be isolated from fasting blood by ultracentrifugation.

As briefly mentioned before, certain apoproteins (in addition to their role as constituents of the lipoprotein molecule) also serve important functions in lipoprotein metabolism (Schaefer et al. 1978). Thus, apo C-II and apo A-I are cofactors for lipoprotein lipase (LPL) and lecithin: cholesteryl acyltransferase (LCAT), respectively. These are key plasma enzymes essential for the lipolysis of triglyceride-rich lipoproteins (LPL) and for cholesterol esterification (LCAT). Apoprotein B in low density lipoproteins (LDL) binds specifically to cell receptors, allowing uptake and intracellular metabolism of cholesteryl esters. In this manner, it regulates plasma and intracellular cholesterol concentration (Goldstein and Brown 1977). Apoprotein E in remnant lipoproteins is recognized by hepatic receptors and thus is essential for the uptake of remnants by the liver.

Lipoproteins can be segregated into four major classes on the basis of their hydrated density (as determined by flotation in the ultracentrifuge), size, or electrophoretic mobility. Triglyceride-rich lipoproteins with a density (relative to water) of <0.95 do not migrate on electrophoresis and are primarily of intestinal origin; these are called chylomicrons. Triglyceride-rich lipoproteins of density 0.95-1.006, which exhibit pre-beta electrophoretic mobility and are primarily of hepatic origin, are designated VLDL. Lipoproteins isolated at a density of 1.006-1.063 are called LDL. LDL have beta electrophoretic mobility, are rich in cholesteryl esters, and are formed exclusively in plasma as a product of VLDL catabolism (Schaefer et al. 1978). The remaining major class, the HDL, are isolated at a density of 1.063-1.216; these are rich in protein and phospholipids and have alpha electrophoretic mobility.

Lipoprotein Lipase (LPL)

The major enzyme involved in the metabolism of triglyceride-rich plasma lipoproteins is LPL, which hydrolyzes the triglycerides of chylomicrons and VLDL. During metabolism, chylomicrons become depleted of triglycerides and enriched in cholesteryl ester. These particles (now called chylomicron remnants) are cleared by the liver as intact particles, presumably by a receptor-mediated process (Floren and Nilsson 1977; Redgrave 1970).

Lecithin: Cholesterol Acyltransferase (LCAT)

LCAT is synthesized by the liver and secreted into the blood, where it plays a major role in lipoprotein metabolism by virtue of its function in cholesterol esterification. Evidence for the hepatic origin of LCAT is its recovery in isolated hepatic perfusates, its absence in hepatectomized animals, and the LCAT deficiency that occurs in many types of human and experimental liver injury (Sabesin et al. 1975, 1977; Sabesin, Kuiken, and Ragland 1978). The basic function of LCAT is the transfer of a fatty acyl group, usually polyunsaturated, from the phospholipid lecithin to unesterified cholesterol, to form cholesteryl esters. LCAT is responsible for the formation of nearly all of the cholesteryl esters in the blood using the newly formed (nascent) discoidal HDL from the liver and intestine as its principal substrate. As a result of its action, nascent HDL is converted to cholesteryl ester-rich, spherical HDL (Tall and Small 1978).

Hepatic Triglyceride Lipase

The mechanism by which chylomicron remnants are removed by the liver and the remaining triglyceride in the remnants hydrolyzed is uncertain. A lipolytic enzyme called hepatic triglyceride lipase, present in the liver, with activity against mono-, di-, and triglycerides, has been postulated to have a role in both processes (LaRosa et al. 1972).

High Density Lipoprotein Uptake

Hepatic Kupffer cells function actively in HDL degradation, accounting for more than 50 percent of the liver's capacity for HDL protein breakdown and cholesteryl ester hydrolysis (Van Berkel et al. 1977). Binding of HDL to hepatocytes may be related to a hepatocyte membrane receptor, and liver lysosomes may be the principal site of HDL proteolytic degradation, just as they appear to be for chylomicron remnants.

Overview of the Liver and Lipoprotein Metabolism

Plasma lipoproteins are constantly in a state of synthesis and degradation. Their metabolism is dependent on complex enzymatic reactions and transfer and exchange of lipids and apoproteins between each class of particles. During this process, nascent lipoprotein particles acquire new apoproteins, cholesteryl esters are synthesized, triglycerides hydrolyzed, and thus, lipoproteins, of drastically different composition and structure, are formed. After chylomicrons enter the lymph, they acquire the apo C peptides, perhaps by transfer from HDL. The acquisition of apo C-II initiates lipolysis of the triglycerides' core of chylomicrons. Concomitant with triglyceride lipolysis is loss of phospholipid, some unesterified cholesterol, apo A-I, and apo C from the chylomicron surface. These lipid and apoprotein losses leave a chylomicron remnant containing apo B, cholesteryl esters, and some residual triglyceride, which are then cleared by the liver. The triglycerides in VLDL are also hydrolyzed by LPL, and similar transfers of surface lipids and apoproteins occur as described for chylomicrons. VLDL is converted into intermediate density particles (IDL) and then LDL, as additional triglyceride is hydrolyzed, and phospholipid, apo A-I, A-II, and apo C peptides are removed. The resulting smaller particle contains mostly cholesteryl esters and apo B, the constituents of LDL.

When HDLs are secreted by the liver or intestine (nascent HDL), they contain mostly unesterified cholesterol and appear by electron microscopy as bilamellar discs instead of the spherical particles isolated from the blood (Hamilton et al. 1976). Nascent HDL is the substrate for LCAT and is converted from discoidal to spherical particles as the newly formed apolar cholesteryl esters form the particle core and are covered on the surface by apoproteins, unesterified cholesterol, and phospholipid. The cholesteryl esters then are transferred to the VLDL → IDL → LDL pathway, perhaps with apo E and apo C. Thus, nascent VLDL becomes enriched with apo C, and apo C in turn (during catabolism) provides a pool of unesterified cholesterol, phospholipid, and apo C used to replenish HDL. HDL may transport unesterified cholesterol from peripheral cells, thus preventing an excessive accumulation of cellular cholesterol. The acquisition of unesterified cholesterol by HDL also provides the substrate for the LCAT reaction. The LDL are a primary source of cholesterol for extrahepatic cells. Binding of LDL to cells is mediated by receptors specific for apo B. After uptake, the cholesteryl esters of LDL are hydrolyzed by a cholesterol esterase forming free cholesterol, which regulates cell cholesterol content.

Lipid and Lipoprotein Abnormalities in Alcoholic Hepatitis

It is most important to clearly differentiate the alterations in lipoprotein metabolism related to alcohol (in which there is associated liver disease) from the effects of alcohol on lipid and lipoprotein metabolism (which occur in the absence of liver disease). This subject has recently been reviewed by Baraona and Lieber (1979), who point out that the "varieties of alcoholic liver disease probably represent steps of a process that should be considered a continuous spectrum." This progression may well range from initial effects of alcohol metabolism on adipose tissue and hepatic lipid metabolism, to compensatory efforts on the part of the organism to altered lipid metabolism, to frank liver injury and failure with concomitant secondary effects on lipid and lipoprotein synthesis and metabolism. For the past several years, our laboratory has investigated the lipoprotein abnormalities associated with alcoholic hepatitis (Ragland, Heppner, and Sabesin 1978; Sabesin et al. 1977, 1980). The lipid and lipoprotein changes in patients with alcoholic hepatitis are quite different from those that occur following the acute administration of alcohol or from those found in blood samples taken from moderate drinkers or even from alcoholics without evidence of overt liver disease.

The hallmarks of the lipid abnormalities in alcoholic hepatitis are hypertriglyceridemia, sometimes hypercholesterolemia, a marked deficiency of cholesteryl esters, a decrease in the cholesterol concentration in the high density lipoproteins (HDL-C), and alpha-lipoprotein (HDL) deficiency (confirmed by a decrease or absence of the major apoprotein, apo A-I in HDL). From our investigations, we have proposed that the primary defect in alcoholic hepatitis is a deficiency of LCAT activity in plasma (Ragland, Heppner, and Sabesin 1978; Sabesin et al. 1977). As a result of the LCAT deficiency, liver-derived nascent HDL, rich in apo E and unesterified cholesterol, accumulates in plasma. Since plasma cholesteryl esters are deficient in apo C and apo E, proteins are not transferred to the VLDL → LDL pathway, which results in the accumulation of VLDL particles which contain almost entirely apo B. Some triglyceride-rich VLDL is partially hydrolyzed by LPL, resulting in the accumulation of a triglyceride-rich LDL. We feel that one of the best indicators of liver damage associated with alcoholism is a decreased level of HDL-C and apo A-I associated with decreased levels of cholesteryl esters.

Disturbances in plasma lipids and lipoproteins are very frequent occurrences in liver disease of various causes and can be explained, in part, by specific abnormalities in lipoprotein synthesis and metabolism. They may be expressed as simple alterations in plasma lipid concentrations or composition, characteristic lipoprotein electrophoretic alterations, or complex disturbances in lipoprotein metabolism. As will be seen, the investigation of lipoprotein metabolism in alcoholic hepatitis provides a unique means of investigating the fundamental role of the liver in lipoprotein metabolism.

Plasma Lipids

The designation of "alcoholic hepatitis" is applied to a clinical and histopathological disorder characterized by derangements of hepatocellular function secondary to hepatocyte necrosis, inflammation, and steatosis (Galambos 1972; Harinasuta and Zimmerman 1971). Invariably patients with alcoholic hepatitis have indulged in massive ethanol consumption for several weeks before the clinical manifestations of jaundice, weakness, fever, and abdominal pain appear. The specific diagnosis of alcoholic hepatitis is based on histopathological criteria of hepatocyte necrosis, the presence of so-called "alcoholic hyalin" in the cytoplasm, inflammatory exudate of lymphocytes and polymorphonuclear leukocytes, and ballooning of individual hepatocytes by an accumulation of triglyceride droplets.

A characteristic feature of acute alcoholic hepatitis is hypertriglyceridemia. Although the triglycerides are usually modestly elevated in most patients, occasionally massive hypertriglyceridemia occurs. Numerous studies have demonstrated hypertriglyceridemia in acute phases of hepatic injury which gradually returns to normal values with resolution of the disease (Sabesin et al. 1972, 1980). The hypertriglyceridemia is often accompanied by concomitant increases in total plasma cholesterol, but the most striking abnormality is the profound decrease in the percentage of cholesteryl esters. Frequently, it is less than 10 percent, as compared to normal values of about 70 percent. The low level of cholesteryl esters reflects marked deficiency of plasma LCAT activity; as will be discussed later, the inability to generate cholesteryl esters is a key factor in the many other abnormalities of lipoprotein composition and metabolism that occur in alcoholic hepatitis. In patients hospitalized and abstinent from alcohol, plasma lipids gradually normalize, reflecting the restitution of normal hepatic function and the concomitant increase in LCAT activity. It is noteworthy that the period of convalescence may vary from weeks to months, and some patients we have studied maintained severely impaired cholesterol esterification for many months, with only gradual improvement toward normal.

The pathogenesis of hypertriglyceridemia in alcoholic hepatitis is undoubtedly secondary to several abnormalities, including excessive hepatic triglyceride secretion, impaired lipolysis, deranged VLDL catabolism, and possibly defective lipoprotein remnant removal by the liver.

Plasma Lipoprotein Electrophoretic Abnormalities

Patients with alcoholic hepatitis always have lipoprotein electrophoretic abnormalities at the onset of illness and, as with plasma lipids and LCAT activity, these changes gradually normalize with the improvement of hepatic function. The characteristic lipoprotein electrophoretic abnormality is a greatly reduced or absent alpha band, absent pre-beta band, and the appearance of a single, broad, densely staining band with mobility between beta and pre-beta.

The normal equivalence of alpha, beta, and pre-beta bands with HDL, LDL, and VLDL does not hold true in liver disease. Indeed, patients with acute hepatitis with an absent alpha band on electrophoresis have HDL of abnormal composition and morphology (Sabesin et al. 1977), which migrates as a component of the broad beta band. VLDL can be isolated by ultracentrifugation, even though prebeta lipoproteins are absent on agarose electrophoresis in liver disease (Seidel et al. 1972).

It has been suggested that liver injury leads to the production of an apo A-I with altered lipid binding ability to account for all the electrophoretic abnormalities (Seidel et al. 1972); however, support for this hypothesis is tenuous at best. A more plausible explanation is that the abnormal HDL of liver disease represents a nascent HDL (Ragland, Bertram, and Sabesin 1978), which cannot be converted to "normal" HDL in an LCAT-deficient state. As nascent HDL contains predominantly apo E and lacks normal amounts of apo A-I (Hamilton et al. 1976; Ragland, Bertram, and Sabesin 1978), surface charge could be altered, leading to abnormal mobility.

Although absence of alpha and pre-beta bands and a wide, densely staining beta band are characteristic and reproducible features of alcoholic hepatitis, these abnormalities are not pathognomic. They occur in other diseases (such as viral hepatitis) characterized by hepatocellular injury and LCAT deficiency. The persistence of the electrophoretic abnormalities can be used as a simple laboratory guide to evaluate hepatocellular function. Thus, it has been shown that the absence or presence of the alpha band in a series of 57 patients with viral hepatitis was a sensitive prognostic indicator (Thallasinos et al. 1975). Of 11 patients with hepatic encephalopathy, 6 survivors showed evidence of a returning alpha band prior to recovery; in 5 fatal cases, the alpha band never returned. Traces of an alpha band's returning prior to clinical improvement was frequently observed in sequential studies (Thallasinos et al. 1975).

Disturbances of Cholesterol Esterification and LCAT Deficiency

Impaired cholesterol esterification is characteristic of alcoholic hepatitis and other acute hepatocellular disorders. Indeed, this aspect of the lipoprotein derangements of hepatic disease has received much more attention than have the hypertriglyceridemia and abnormal electrophoretic patterns. The level of cholesteryl esters appears to be directly correlated with the plasma LCAT activity. Plasma LCAT activity is sufficient to account for all the plasma-esterified cholesterol. Furthermore, the rate of cholesteryl ester formation in vitro secondary to LCAT activity agrees with the estimated turnover rates of plasma cholesterol in vivo. The importance of the liver in the production of this key enzyme, as well as its cofactor and principal substrate, provides a sufficient basis to explain the disturbances of cholesterol esterification that accompany liver injury. Plasma from patients with liver disease contains increased lecithin and unesterified cholesterol and decreased lysolecithin and cholesteryl ester—findings that are strikingly similar to those found in familial LCAT deficiency (Glomset et al. 1973). Familial LCAT deficiency is characterized by profound deficiency of cholesteryl esters in the absence of liver disease, confirming the negligible role of intrahepatic cholesteryl ester synthesis (Norum and Gjone 1967).

Patients with parenchymal hepatic dysfunction consistently show an LCAT deficiency; the degree of the deficiency generally parallels the extent of liver injury as assessed by clinical and laboratory criteria. The decrease in LCAT activity is variable (even in groups of patients with the same type of liver disease), and this variability appears to reflect the extent of hepatocellular injury (Sabesin et al. 1977, 1978). The prognostic significance of the degree of LCAT deficiency has been emphasized, since patients with low activity seem to have a greater impairment of hepatic function and, therefore, a substantially poorer prognosis than do patients with lesser degrees of impaired LCAT activity. Increasing LCAT and plasma cholesteryl esters occur with clinical recovery in patients with acute parenchymal hepatic injury, which strongly suggests a causal relationship. It is likely that the LCAT deficiency arises as a result of decreased enzyme synthesis and/or release by the damaged liver, since LCAT deficiencies are found in hepatectomized animals and in models of hepatic parenchymal injury arising from treatment with hepatotoxic agents such as galactosamine (Sabesin et al. 1978).

Lipoprotein Compositional and Ultrastructural Abnormalities

We have performed detailed compositional studies of lipoprotein fractions isolated by ultracentrifugation at various intervals throughout the course of illness in patients with alcoholic hepatitis. In general, the most striking compositional abnormality, in each of the fractions, is the profound depression in the percentage of cholesteryl esters. These can remain severely decreased for weeks or even months (depending on the severity of the liver injury) before they gradually begin to return toward normal. In VLDL, the decrease in cholesteryl esters is accompanied by a moderate increase in phospholipids. In addition to the profound depression in the percentage of cholesteryl esters in LDL, this fraction is characterized by a fourfold increase in triglycerides and is also enriched in phospholipids. The HDL fraction is remarkable for the persistent decrease in percentage of cholesteryl esters. Unesterified cholesterol and phospholipids in HDL may be two to three times normal during the active phase of alcoholic hepatitis.

The compositional abnormalities of the lipoproteins are associated with ultrastructural alterations, which can be observed readily by electron microscopy using negatively stained preparations of whole plasma. In whole plasma preparations, a heterogeneous mixture of all of the lipoproteins is visualized. Many of the lipoproteins are bizarre in structure, appearing as bilamellar vesicles and chains of stacked discs of a wide range of sizes. Electron micrographs of negatively stained lipoproteins isolated by sequential ultracentrifugation from plasma during illness and after recovery illustrate the ultrastructural abnormalities. In normal plasma, lipoproteins appear as uniform spherical structures of gradually decreasing size: VLDL, 300 to 750 Å; LDL, 170 to 260 Å; and HDL, 90 to 120 Å. In alcoholic hepatitis, the VLDL fraction contains particles similar in structure and size to normal VLDL. The LDL, isolated at $1.006 < d < 1.063$, contain some spherical particles, similar in appearance and size to normal LDL (mean diameter, 225 Å), although many particles are much larger (500 Å), and (particularly if there is concomitant severe intrahepatic cholestasis) the LDL may appear as bilamellar vesicles and discs measuring 450-500 Å in diameter and 105-130 Å in thickness. The HDL fractions contain some spherical particles of approximately normal HDL size, but most of the lipoproteins in this fraction consist of long chains of bilamellar stacked discs, measuring 150-240 Å in diameter and 30-50 Å in thickness.

Compositional analyses of isolated lipoprotein classes have been performed to determine the distribution of cholesterol and apo A-I, the major apoprotein of HDL. An example of this analysis is the isolation of the concentration of apo A-I and cholesterol in whole plasma and in lipoprotein fractions by sequential ultracentrifugation. The study was performed in a patient who had typical alcoholic hepatitis and who showed considerable improvement over a 3-week period. Initially, apo A-I was almost absent in whole plasma and in HDL, total cholesterol was decreased, and HDL-C was severely depressed. This was associated with marked impairment of cholesterol esterification. With clinical improvement apo A-I increased in the HDL, and the HDL-C concentration improved tenfold.

These abnormalities in HDL composition were reflected in the morphology of the HDL particles, which appeared initially as chains of bilamellar discs; however, with partial and then almost complete recovery, the discoidal HDL disappeared and were replaced by uniform spherical particles.

To further define the abnormalities in HDL in alcoholic hepatitis, a density gradient separation of LDL plus HDL was performed. The results obtained from the density gradient separation of the LDL plus HDL fraction are summarized as follows. At the beginning of the illness, a cholesterol-rich peak was found at the top of the gradient in the LDL density range. It contained most of the cholesteryl ester present in the gradient, along with increased amounts of apoprotein B (as compared to gradients from normal samples). No cholesterol peak was found in the portion of the gradient representing HDL$_3$ and HDL$_2$, nor was any significant amount of apo A-I present in any of the gradient samples. With recovery, and with the return of LCAT activity, cholesteryl esters in the LDL, HDL$_2$, and HDL$_3$ fractions gradually increased, and apo A-I appeared in the HDL$_2$ and HDL$_3$ fractions.

Apoprotein Composition in Alcoholic Hepatitis

The apoproteins of isolated VLDL, LDL, and HDL were analyzed at varying intervals during the course of hospitalization of patients with alcoholic hepatitis. A typical profile of the composition and concentration of the apoproteins obtained during the month of hospitalization of a typical patient with alcoholic hepatitis is as follows. On admission, VLDL contained 97 percent apo B, but, unlike normal, the C peptides and apo E were nearly absent. LDL contained more than 92 percent apo B at all times, similar to normal LDL; however, the apoprotein content of HDL was strikingly abnormal. Normal HDL contain apo A-I, apo A-II, the apo C peptides, and a trace of apo E. In contrast, the predominant apoprotein in alcoholic hepatitis HDL was apo E, and apo A-I was deficient.

As the patient improved, the apoprotein composition of the VLDL and HDL gradually returned toward normal. This was associated with a gradual increase in plasma LCAT activity and an increase in the percentage of cholesteryl esters in plasma. The return toward normal of the apoproteins in the isolated lipoprotein fractions was characterized by a gradual decrease in apo E in HDL and an increase in apo A-I. The relative proportion of apo B in VLDL gradually decreased; apo C and apo E gradually increased.

Lipoprotein Metabolic Derangements in Alcoholic Hepatitis

From the preceding discussion, it can be seen that alcoholic hepatitis is associated with many abnormalities in the concentration and composition of blood lipids and lipoproteins. The pathogenesis of these changes is undoubtedly multifactorial, reflecting complex biosynthetic, enzymatic, and catabolic derangements in lipoprotein catabolism. Central to these are the effects of alcohol on lipid metabolism and the deleterious effects of alcohol on hepatocellular function. These result in excessive hepatic secretion of triglyceride-rich VLDL; but, because of associated apoprotein biosynthetic defects, LCAT deficiency and possibly defects in triglyceride lipolysis, remnant removal, and/or hepatocyte triglyceride lipase deficiency, striking derangements in lipoprotein metabolism occur. The results obtained in alcoholic hepatitis are compatible with the hypothesis that the liver secretes primarily two lipoprotein types, nascent HDL whose major apoprotein is apo E, and nascent VLDL whose major apoprotein is apo B. Normally, nascent HDL is acted on by LCAT, and the cholesteryl esters formed are transferred to the nascent VLDL → LDL pathway along with apo E and apo C. As a result of LCAT activity, HDL containing primarily apo A-I is the major component of the HDL fraction. In alcoholic hepatitis, in which there may a near absence of LCAT in plasma, nascent HDL is not metabolized and accumulates in plasma. As a result, cholesteryl esters are not formed and thus not transferred to the VLDL → LDL pathway along with apo E and apo C. Therefore, nascent VLDL—with an essentially normal lipid composition, but containing primarily apo B—accumulates. Apparently, nascent VLDL is partially degraded by the action of lipases, resulting in the accumulation of a triglyceride-rich fraction of density (1.006 to 1.063) and containing primarily apo B; but its further metabolism to IDL and LDL is blocked because of the lack of cholesteryl esters and possibly also by the absence of apo E and apo C.

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Blood Serum Lipids in a Group of Chronic Alcoholic Patients in Poland

Ignacy Wald

As reviewed by Baraona and Lieber (1979), alcoholic hyperlipemia is a common, transient response to ethanol intake, but some individuals manifest unusual sensitivity to the hyperlipemic effects of ethanol. On the other hand, hyperlipemia may be considered as a protective mechanism against the development of fatty liver; as the hepatic injury increases, liver becomes unable to dispose of lipids as serum lipoproteins, especially the triglyceride-rich ones. The following presents some results obtained in the Psychoneurological Institute in Warsaw by Dr. Hanna Wehr and her coworkers (1979).

Serum lipids in alcoholic subjects (patients of an alcohol ward in Warsaw) were studied in order to establish the frequency of alcoholic hyperlipemia in this population. The group of patients consisted of 92 men, aged 18-59, and the control group of 45 blood donors.

Venous blood was drawn after overnight fasting and 48 hours after alcohol withdrawal; serum triglycerides and cholesterol levels were then determined.

Only 5 of 92 alcoholic patients had markedly increased levels of serum lipids (triglycerides or cholesterol or both). These five represented hyperlipoproteinemia type IIa (hypercholesterolemia without concomitant triglyceride rise) in two cases and hyperlipoproteinemia type IV (hypertriglyceridemia) in three of them.

Many patients had low serum triglyceride concentrations—in 35 patients, below 70 mg/% and in 22 patients, lower than 50 mg/%.

The large variation of serum triglyceride levels in alcoholic patients is shown in table 1. The mean value of triglyceride levels for the whole group of alcoholic subjects was significantly lower as compared with an age- and sex-matched control group. A small difference in cholesterol level was not significant. The evaluaton of the results by the nonparametric Mann-Whitney U test is presented in table 1.

In some of the drinkers, the effect of ethanol on serum triglycerides was also followed over 2 days following the intoxication state. Various types of responses are shown in figure 1. The first pattern represents alcohol-induced hypertriglyceridemia: Extremely high values were

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observed shortly after drinking, diminishing after alcohol withdrawal but remaining still markedly high 48 hours after cessation of drinking. In the second, no trace of lipemic response could be observed—triglyceride level constantly remained very low. The third type appeared as an intermediate pattern—a moderate triglyceride level, lowered somewhat within 2 days after alcohol withdrawal.

The prevalence of established hyperlipoproteinemia in the examined group of alcoholic patients was within the range of that observed in the whole population.

In many drinkers triglyceride levels were low. They probably represented the group with the more advanced impairment of liver function.

In epidemiologic studies (Yano et al. 1977), higher levels of HDL cholesterol were observed in the moderate drinkers as compared with the abstainers. It has not been established whether the same pertains to heavy drinkers with low serum triglyceride levels; a study has been initiated to examine this question. A more detailed study concerning the apo-lipoprotein changes in chronic alcoholism is also in preparation.

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Figure 1. Examples of Serum Triglyceride Patterns After Ethanol Ingestion by Alcoholic Patients

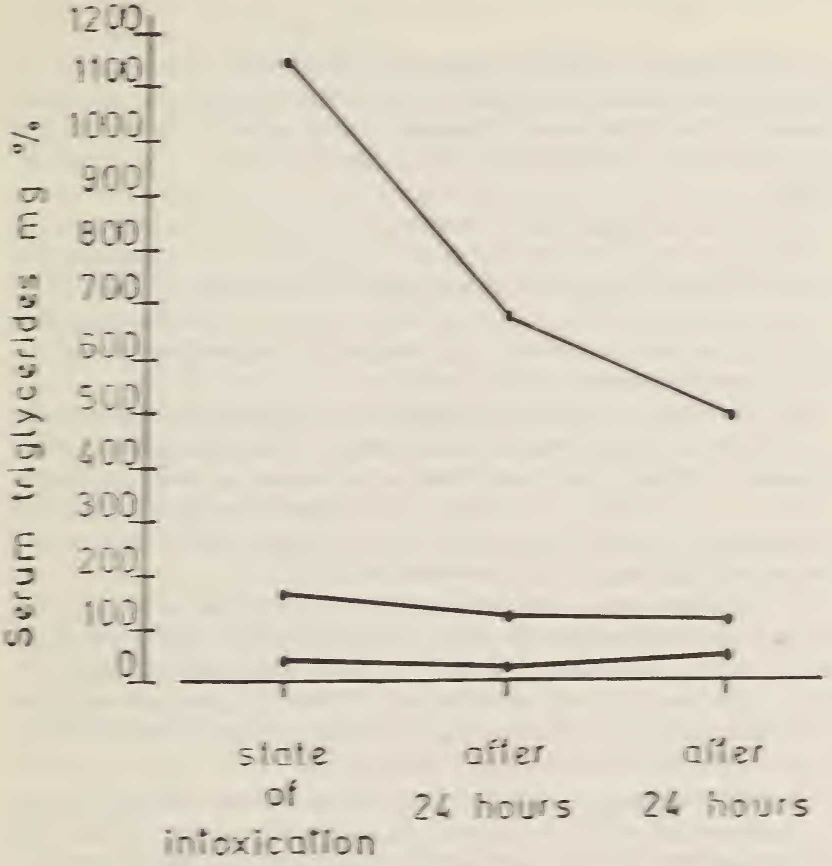

Table 1. Evaluation of the Results of Triglyceride and Cholesterol Determination

Value	Alcoholic subjects	Substance	Controls
		Triglycerides	
n	91		44
Mean value ± SD	160.5 ± 72.3		114.0 ± 42.3
p		0.0046	
		Cholesterol	
n	92		44
Mean value ± SD	220.1 ± 44.3		205.7 ± 45.3
p		0.2386	

Ethanol and Bile Acids

Andre Lefevre

In the sixties, it had been observed that chronic administration of ethanol produces an accumulation of esterified cholesterol in the liver, as well as an increase in cholesterol concentration in the blood; this was found in rats (DeCarli and Lieber 1967) and in men (Lieber et al. 1963).

Since the diet given with the ethanol was devoid of cholesterol, it could be assumed that simultaneous accumulation of cholesterol in the blood and in the liver resulted mainly from increased synthesis or decreased cholesterol disposition, or both, and it was thus reasonable to study the effects of alcohol on bile acids since they represent the major pathway of cholesterol degradation.

Dr. Lieber and I showed that chronic administration of ethanol prolonged the excretion time of labeled cholic or chenodeoxycholic acids, increased slightly the pool size, and decreased daily excretion (Lefevre et al. 1972). By contrast, supplementation of the diet with cholesterol shortened the excretion time, did not modify pool size, and increased daily excretion (Lefevre et al. 1972).

When ethanol and cholesterol feeding were combined, the effects of ethanol prevailed, and there was suppression of the adaptive changes in bile acid metabolism induced by cholesterol feeding (figure 1). There was also a greater accumulation of esterified cholesterol in the liver than that produced by cholesterol alone, ethanol administration alone, or the summation of both effects.

In 1977, Lakshman and Veech studied the effects of alcohol on the 7α-hydroxylase, which is the rate-limiting enzyme step in bile acid synthesis. The hydroxylase activity was not significantly affected within 2 hours after a single intraperitoneal injection of ethanol. By contrast, chronic ethanol feeding for 21 days caused a 56-percent decrease in cholesterol 7α-hydroxylase activity at the diurnal high point, without affecting the diurnal rhythm of the enzyme.

In 1978, Lakshman et al. performed in vivo experiments in bile duct cannulated rats and confirmed that chronic administration of ethanol caused a 50-percent inhibition in the rate of incorporation of [4-^{14}C] cholesterol to bile acids.

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Evidence from the above studies suggests that the major defect causing ethanol-induced cholesterol accumulation in the liver results from decreased degradation of cholesterol into bile acids. The precise mechanism, however, by which chronic ethanol feeding affects bile acid synthesis, is far from clear.

It should be remembered that control of bile acid synthesis is an intricate phenomenon, involving not only the liver but also the gut, which, in the rat, contains more than 90 percent of the bile acid pool. Actually, bile acid synthesis is believed to be regulated by the size, composition, and circulation time of the bile acid pool, as a result of negative feedback of bile acids returning to the liver via portal circulation.

Which one among these factors is affected by chronic ethanol administration?

Since the 7α-hydroxylase and the microsomal ethanol oxidizing system (MEOS) are located in the microsomal system and have similar cofactors (such as NADPH and cytochrome P-450), it is conceivable that bile acids and ethanol are competing with each other. Until now, however, no experiment has been reported to confirm or invalidate this hypothesis.

It should be pointed out that several characters of the 7α-hydroxylase system are different from those of the drug oxidation system. Using various inducing agents (such as clofibrate and cholestyramine) in young rats, Mellon and coworkers (1978) studied the hepatic metabolism of several drugs in parallel with the 7α-hydroxylase system. They concluded that the evidence for the similarity of the mixed-function oxidase system (which participates in drug oxidation) and the system that catalyzes the hydroxylation of cholesterol is somewhat equivocal. Similarly, Deliconstantinos and his group (1980) studied the effects of estradiol treatment on the 7α-hydroxylase and drug oxidation systems in castrated rats and their results suggest that the cytochrome P-450 participating in the cholesterol 7α-hydroxylation is different from that catalyzing the metabolism of drugs and xenobiotics.

On the other hand, it is also quite possible that ethanol influences bile acid synthesis in some indirect ways, for example, by modifying intestinal motility. Hardison, Tomaszewski, and Grundy (1979) have observed, in men, that sorbitol shortened transit time by 38 percent and increased hourly bile acid excretion rate by 31 percent. Atropine increased transit time by 38 percent and decreased hourly bile acid excretion rate by 38 percent. Their observations, however, were made during acute experiments, and it is possible that, in chronic studies, bile acid synthesis may remain constant following adaptaton of the bile acid pools to modification of the cycling frequency.

As a second example, Goebell and his group (1980) have studied the bile acid output in cats in response to intravenous ethanol before and after bile acid depletion of the small bowel. They observed that acute infusion of alcohol more than doubled bile acid absorption from the gut. These are preliminary experiments, but, if confirmed, this ethanol effect could play a significant role in bile acid synthesis.

It has been known for many years that modification of the intestinal bacteria can influence the whole pattern of bile acid metabolism. Studies on gut microflora are entangled with deceptions, but Bode and his group (1980) have reported some quantitative as well as qualitative changes of the intestinal flora in patients with or without alcoholic liver disease. It is important, however, to dissociate the effects of ethanol from the consequences of the hepatic lesion, and it is still probably too early to draw firm conclusions from this report.

We have limited ourselves to the effects of acute or chronic ethanol administration on bile acid metabolism, but it should be remembered that bile acid pattern may be altered by hepatic lesions (not necessarily alcohol-induced).

Whereas possible bile acid alterations are not well known in the course of alcoholic hepatitis, the abnormalities of bile acid metabolism in cirrhosis have been extensively studied for many years (Carey 1958, Sjowall 1960, and Vlahcevic et al. 1972). It has been demonstrated that cholic acid synthesis is reduced in early cirrhosis and that this decrease is proportional to the severity of the disease. Chenodeoxycholic acid synthesis, however, was much less sensitive to these pathological changes (McCormick et al. 1973). According to recent studies, these pool modifications result from alteration of the 12α-hydroxylation system, whereas the side chain oxidation and the 7α-hydroxylation systems are only slightly affected (Patteson et al. 1980).

Once again, the hepatic lesion is not the only culprit: Alterations in metabolism of the secondary bile acid (such as deoxycholic acid) suggest that changes in the fecal bacterial flora are again playing a definitive role in modifying bile acid metabolism (Yoshida et al. 1975).

In summary, the hypothesis that chronic administration of ethanol decreases bile acid synthesis is supported by several observations. The mechanisms of this action may combine various alterations and are not yet well understood. Furthermore, these mechanisms may become quite intricate when ethanol-induced hepatic (and intestinal ?) lesions contribute by themselves to alterations in bile acid metabolism.

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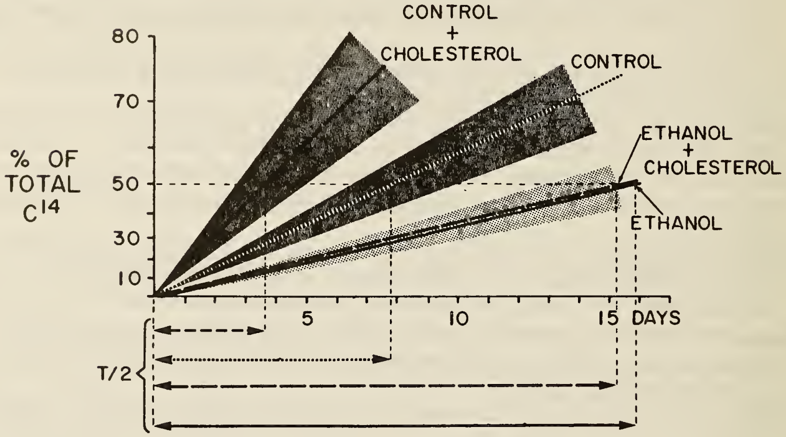
Figure 1. Cumulative Excretion in Feces of Labeled Bile Acids Following an Intraperitoneal Injection of (14C) Cholic Acid



Note: Shaded areas represent SEM.

Alcoholic Triglyceridemia Related to Stage of Disease*

Stephen A. Borowsky

Abstract

This paper provides an historical perspective and reviews studies of triglyceridemia in normal subjects and alcoholics. According to the author, hypertriglyceridemia in alcoholic liver disease appears to be related to stage of disease and fat intake in its magnitude and duration; the lipoprotein character of triglyceridemia changes with the advance of liver disease from fatty liver to cirrhosis. In patients with fatty liver there is increased production of very low-density lipoproteins (VLDL); in patients with cirrhosis there is retention of chylomicrons in the blood. Current studies indicate the retention of chylomicrons may be related to deficiency of apo lipoprotein C II, a protein produced by the liver, the author concludes.

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SESSION VII: ENDOSKELETON AND EXOSKELETON INCLUDING COLLAGEN

Endoskeleton and Exoskeleton Including Collagen*

Marcos Rojkind

This roundtable was organized to discuss a heterogenous group of macromolecules located inside the cell, on the plasma membrane of the cells, or in the extracellular matrix, which are involved directly or indirectly in determining the shape and structure of the cells. Some of the proteins may also play a role in determining or modifying cell function.

The intracellular proteins are represented by the group of contractile proteins (actin, myosin, and so on), the microtubular proteins, and the 10 nm microfilaments. The membrane macromolecules include proteins such as spectrin, the major glycoprotein of red cell plasma membrane, and a group of proteins (collagens, fibronectin) and glycosaminoglycans that are present both at the plasma membrane and as part of the extracellular connective tissue of the liver. Doctors Feinman, Hahn, Henley, Okasaki, and I will be discussing mainly the problems related to the nature of the extracellular matrix and its modifications in cirrhosis, the cells involved in the biosynthesis of the connective tissue proteins and in the contraction of the scar, and the role of collagen synthesis and collagen degradation in the overall process of collagen accumulation in liver cirrhosis. Dr. French will analyze the relationship between intermediary filaments and alcoholic hyaline.

The Nature of the Extracellular Matrix

The extracellular connective tissue of the liver is composed of three groups of macromolecules: collagens, glycosaminoglycans, and glycoproteins. A summary of their chemical nature, properties, and liver distribution is presented in tables 1, 2, and 3.

Collagens

Four of the five collagen types so far described in animal tissues,

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NOTE: Figures and tables appear at end of paper.

* The original work of this manuscript was supported by CONACyT grants 1576 and 790136.

mainly types I, III, IV, and V, have been found in the liver. Type I collagen corresponds to the heavy collagen bundles of the liver, and it constitutes approximately 33 percent of total liver collagen. It is located in portal triads and in large vessels. Occasionally, type I collagen fibers are detected inside the liver lobule. Type III collagen is represented by some but not all of the reticulin fibers of the liver. In general, type III is mixed with type I collagen in the portal triads and around the large vessels. It is also present inside the liver lobule, sometimes in close contact with the cellular processes of the Ito cells. In the normal liver, type III collagen is present in equimolar concentrations with type I collagen; the ratio of I:III collagens is 1.

Type IV collagen is the basement membrane collagen; it is present in about 10 percent concentration. It is localized in the portal tract beneath the endothelial lining of hepatic arteries, of portal veins and lymphatic vessels. It is also present around smooth muscle cells in the vessel wall and around bile duct and ductules. Inside the liver lobule, type IV collagen is found along the sinusoids between the endothelial lining and liver cell plates.

Type V collagen accounts for about 10 percent of total liver collagen. It is present in some places where basement membrane collagen is localized. However, it has been suggested that this type of collagen may be part of the exoskeleton, and thus it is in close association with the sinusoidal surface of the hepatocytes. Type V collagen is also present in portal triads and in terminal venule zones.

Glycosaminoglycans

The glycosaminoglycans present in the normal liver are 10 percent hyaluronic acid, 7 percent dermatan sulfate, 7 percent chondroitin sulfates, and 75 percent heparan sulfate. The latter is the major glycosaminoglycan of liver cell plasma membrane. The distribution of each type of glycosaminoglycan within the liver is not known.

Glycoproteins

Although the extracellular connective tissues contain a large variety of glycoproteins, only two have been characterized. One of the proteins is laminin; it was originally isolated and characterized from a murine tumor synthesizing basement membrane. In the liver, laminin is co-distributed with type IV collagen, except for its distribution between the endothelial lining and liver cell plates. Laminin may play an important role in morphogenesis and may serve as an attachment factor for some epithelial cells in culture.

Fibronectin is the other well-characterized glycoprotein found in the liver. It is similar in immunologic reactivity, and amino acid and sugar composition to the cold-insoluble globulin found in plasma. It is the major glycoprotein of fibroblast plasma membrane, and it is missing in transformed cells. Fibronectin is present in the liver along the sinusoids between endothelial cells and liver plates. It is co-distributed in several places with type IV collagen except in the periportal regions. Fibronectin serves as an attachment factor for fibroblasts and macrophagic cells in culture and can promote changes in the phenotypic expression of some mesenchymal cells. For example, chondroblasts will produce an abnormal extracellular matrix, when incubated in the presence of cold-insoluble globulin.

The Nature of the Scar

In liver cirrhosis, all collagen types are increased. In livers containing less than 20 mg of collagen per g of fresh tissue, the ratio of type I to type III collagen is maintained between normal limits. However, in livers containing more than 20 mg of collagen per g of fresh tissue, type I collagen predominates, and the ratio I:III is increased. The distribution of collagens in the liver is also modified in cirrhosis. All types of collagen are found mixed together in the portal tracts, inside the liver lobule, and in the collagen bands that surround the so-called regenerative nodules.

Fibronectin and laminin are also increased. However, this statement is based on immunofluorescent studies performed with cirrhotic livers, and there are no quantitative data. Recently, it has been shown that, in granulation tissue, the production of fibronectin precedes the synthesis of type III collagen. It has also been suggested that the silver-staining properties of type III collagen are indeed related to the fibronectin associated with it.

Increased Deposition of Liver Collagen

Increased deposition of liver collagen could be the result of increased collagen biosynthesis, decreased collagen degradation, or both. Collagen synthesis has been determined in vitro with human and animal liver slices. In all instances studied, collagen synthesis is increased several-fold above control values. Collagen degradation, on the other hand, has produced conflicting results. Measurements of collagenase activity in cirrhotic livers can be normal, increased, or decreased. It has been shown that, in early stages of liver fibrosis, collagenase activity is increased and, in late stages, the activity of this enzyme decreases. Immunofluorescence studies with anticollagenase antibodies have revealed that the enzyme is present around all reticulin and collagen fibers in the normal liver. In cirrhotic liver, collagenase is present only on the outer part of the heavy collagen fibers, and it is missing in the reticulin fibers inside the lobule. In advanced liver fibrosis, the enzyme is absent. It has been suggested that the lack of collagen degradation could be related to the presence of inhibitors. Perhaps the most important factor in the lack of collagen degradation is the three-dimensional organization of the scar. The enzyme may have access only to the most external portions of the collagen fibrils. The heavier the collagen bundles (the more type I collagen), the lesser digestion.

Metabolic Alterations Associated with Increased Deposition of Collagen

Several metabolic alterations in the liver have been associated with increased liver fibrosis. They occur in addition to the changes in enzymes that participate directly in the posttranslational modifications of collagen. In all types of liver fibrosis studied, the pool of free proline in the liver is increased two-fold. This increase is due to increased degradation of proline by the mitochondrial enzyme, proline oxidase. In alcoholic patients, in addition to the elevation in liver proline, serum proline and blood lactic acid are also increased. Lactic acid is a potent inhibitor of proline oxidase and thus could contribute to the elevation in serum proline (figures 1 and 2).

Although it has been suggested that elevations in blood lactic acid are directly related to alcohol metabolism by alcohol dehydrogenase, recent evidence suggests that lactic acid elevations already imply an alteration in liver function of the alcoholic patient. It is also possible that hyperlactacedemia in the alcoholic indicates his or her genetic predisposition to develop cirrhosis. Only about one-third of the alcoholics have cirrhosis.

In experimental models of liver cirrhosis and in human schistosomiasis, it has been shown that liver collagen synthesis is directly related to the concentration of free proline in the incubation medium. In all instances, linear rates of collagen synthesis are obtained in vitro with proline concentrations of approximately 0.45 mM. In cirrhotic livers, the pool of free proline is 0.4 mM; thus, the amount of proline in the liver is sufficient to maintain maximum rates of collagen synthesis.

In the experimental model of CCl_4-induced cirrhosis, all the aforementioned changes are completely reverted within 2 weeks after discontinuation of the administration of the toxic agent. This suggests that perhaps in human liver disease all the alterations are reversible after discontinuation of the agent (chemical or biological) that started the fibrogenic response. However, in the instances in which antibodies have been produced to membrane lipoproteins, to viral antigens, or to proteins that accumulate in the liver (alcoholic hyaline), the destruction of liver cells will continue. This self-perpetuation mechanism could further stimulate the production of collagen and the development of liver cirrhosis.

Cells Involved in the Fibrogenic Response of the Liver

It has been established already that all types of cells in culture, including adult rat hepatocytes, are capable of producing different collagen types. However, the actual contribution of each individual cell to the overall deposition of liver collagen is not known. Perhaps one of the most important observations is that myofibroblasts are present in the heavy collagen bands surrounding the regenerative nodules. These cells possess a contractile apparatus and have been shown to be responsible for the contraction of scar tissue during wound healing. These cells could also participate in the contraction of the scar in the liver. Accordingly, the regenerative nodules could be formed by active contraction of the scar and squeezing of the cells inside the nodule and not, as previously believed, by active regeneration of cells trapped inside the fibrous tissue. This result could explain, in part, some of the beneficial effects of colchicine in the treatment of liver cirrhosis, since colchicine could inhibit the contraction of the myofibroblasts.

Nonaggressive Parameters of Liver Fibrogenesis

Collagen is synthesized in a manner similar to that of other export proteins, and it contains (at both the amino-terminal and carboxyl-terminal ends) nonhelical peptide extensions. These are cleaved during the processing of the intracellular procollagen to the extracellular fibrillar collagen. The amino-terminal peptides have an apparent longer half-life than do the carboxyl peptides, and they have been detected in some biological fluids with the use of a very sensitive radioimmunoassay. The peptide is further degraded, and smaller fragments (still containing antigenic determinants) have been detected in the urine.

The determination of type III procollagen amino-terminal peptide is currently being evaluated as a parameter of liver fibrogenesis. The results suggest that the measure may be a useful parameter. However, the source of the peptide still remains to be determined. It has been suggested that the peptide in the blood is derived from de novo collagen synthesis in the liver. However, intact procollagen type III molecules have been detected in the liver, and the serum peptide could be derived from the breakdown of tissue procollagen. Independently of the source of the peptide, the usefulness of this technique must be evaluated, since the results obtained so far are quite promising.

Lactic acid and blood proline values could also provide further information about liver fibrogenesis in alcoholics. This method is currently being evaluated by my colleagues at the Instituto Nacional de la Nutrición in México.

Measurements of serum prolylhydroxylase activity are meaningless, because (1) the enzyme is unstable and (2) there are inhibitors present in the sera of some patients. In this respect, the determination of serum immunoreactive prolylhydroxylase is more promising, because there is a correlation between liver prolylhydroxylase activity and serum immunoreactive protein. None of the methods described is a substitute for a liver biopsy.

Connection of Liver Injury with Liver Fibrogenesis

Liver injury could trigger liver fibrogenesis by the stimulation of an inflammatory and/or immunologic response of the host. The activation of Kupffer cells in the liver could elicit the production of a large variety of substances (kinins, nectins, prostaglandins) that could induce migration and proliferation of fibroblasts and perhaps also of myofibroblasts. In addition, some of the factors could also enhance the production of collagen by the different populations of cells. It has been shown that fibronectin, a glycoprotein produced by Kupffer cells, is chemotactic for fibroblasts and that it can modify the phenotypic expression of some cells in culture.

Independently of the mechanism that initially produces liver damage, it appears that animals, including man, have a limited way to respond to injury. They show either regeneration, healing, or a combination of both. In acute liver disease (when the damage produced is extensive), the sudden decrease in functional parenchyma could stimulate liver cell regeneration.

In cases of acute liver failure, if the patient survives, complete restoration of liver architecture and function will be obtained. However, if the cells destroyed are few, and the stimulus is repeated, inflammation and wound healing will prevail; thus, a net deposition of collagen will occur. Accordingly, the nature of the scar will always be the same, as already demonstrated, independent of the large variety of etiologic agents that can produce liver cirrhosis.

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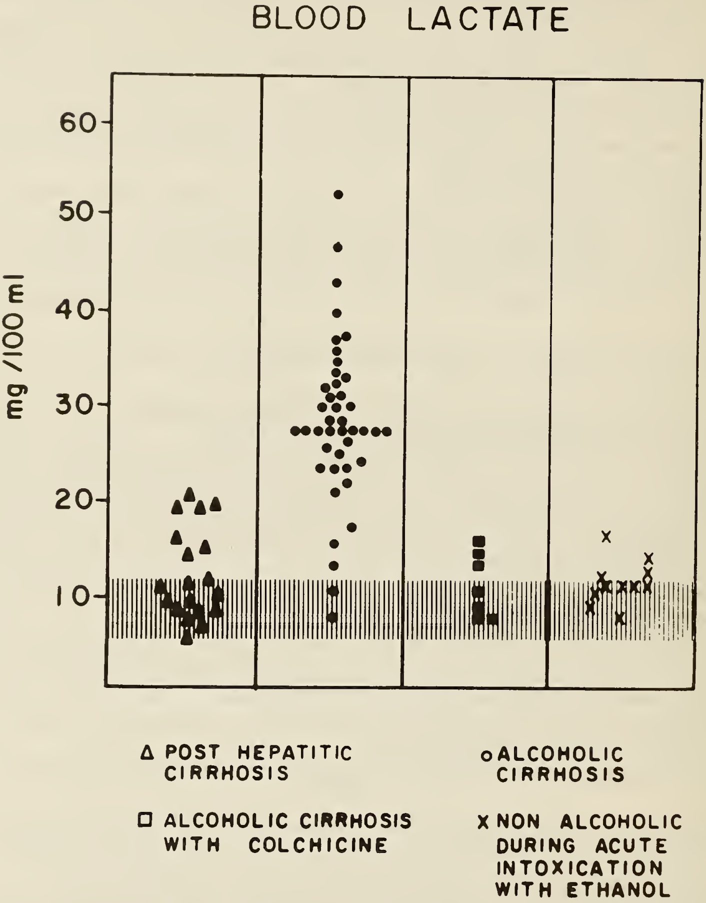
Figure 1. Blood Lactate Concentrations

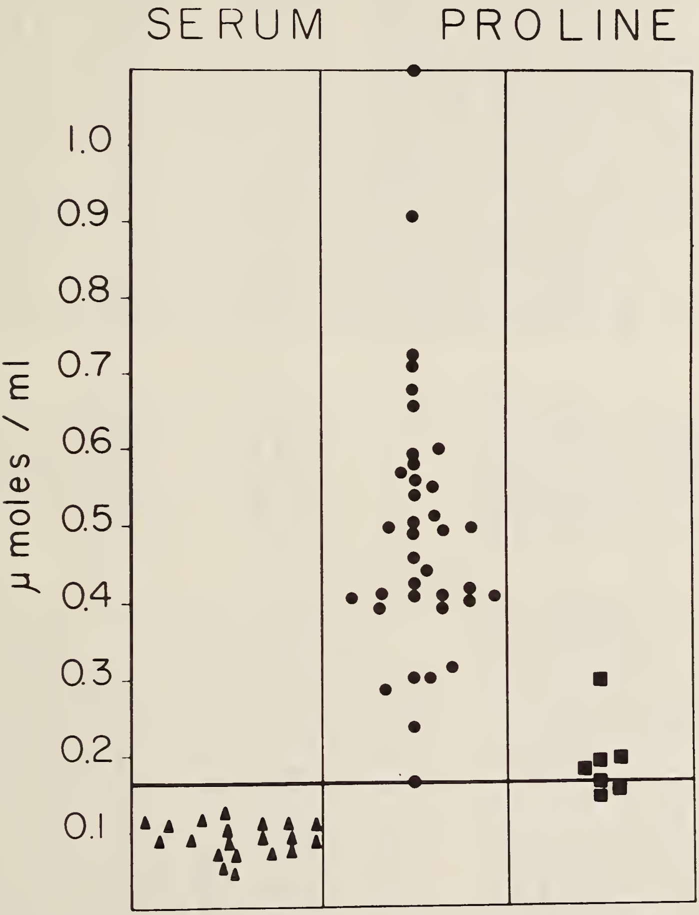


Blood lactate concentration in 20 patients with posthepatic cirrhosis, 42 patients with alcoholic cirrhosis, 7 patients with alcoholic cirrhosis treated wiith colchicine (1 mg/day, 5 days/week) for 1 year, and 11 nonalcoholic subjects 3 hours after acute intoxication with ethanol. The shaded zone represents the mean value of normal subject ± two standard deviations of the mean.

▲ Posthepatic cirrhosis
• Alcoholic cirrhosis
■ Alcoholic cirrhosis with colchicine
X Nonalcoholic subjects during acute intoxication with ethanol.

(From Kershenobich, D.; García-Tsao, G.; Alvarez Saldaña, S.; and Rojkind, M. Relationship between blood lactic acid and serum proline in alcoholic liver cirrhosis. *Gastroenterology*, 80:1012-14. 1981. Copyright 1981 by Elsevier North-Holland, Inc. [NY].)

Figure 2. Serum Proline Concentration in Cirrhotic Patients



(See legend of figure 1). The horizontal line represents the mean value previously reported for our normal subjects. Proline values of nonalcoholics during acute intoxication with ethanol (not shown) did not differ form the controls.

▲ Posthepatic cirrhosis) (•-- Alcoholic cirrhosis)
■ Alcoholic cirrhosis with colchicine.

(From Kershenobich, D.; García-Tsao, G.; Alvarez Saldaña, S.; and Rojkind, M. Relationship between blood lactic acid and serum proline in alcoholic liver cirrhosis. *Gastroenterology*, 80:1012-1014. 1981. Copyright 1981 by Elsevier North-Holland, Inc. [NY].)

Table 1. Collagen Components of Liver Extracellular Matrix

Collagen Type	Molecular Formula	Special Features	Tissue Distribution	Liver Localization
I	$\{\alpha 1(\mathrm{I})\}_2\alpha 2$	Lys+hylys=35 residues; hylys=7. Low glycosylation. Heavy collagen bundles of most tissues. Solubilized with pepsin and precipitable from neutral pH buffers (Tris HCl) with 2.5 M NaCl.	All connective tissues: skin, tendon, bone, dentin, liver, lung, heart, fascia, etc.	Heavy collagen bundles in portal triads and vascular regions; occasionally seen in controlobular areas. Forms the dense connective tissue matrix. Its concentration in normal liver is approximately 2 mg/g of fresh tissue and the ratio type I/type III is 1. Its concentration in cirrhotic liver is about 15 mg/g of fresh tissue. The ratio I/III increases to > 2.0. It forms the dense connective tissue matrix of the scar.
I-trimer	$\{\alpha 1(\mathrm{I})\}_3$	Present in tissues synthesizing collagen after injury or inflammation. Produced by "aged" or dedifferentiated cells. Solubilized with pepsin and precipitable from Tris HCl buffer with 4.0 M NaCl.	Skin, cartilage, liver, gingiva, chondrocytes.	Present in very small concentration in normal liver and increases in cirrhotic livers. Its distribution is not known.
III	$\{\alpha 1(\mathrm{III})\}_3$	Lys+hylys=35; hylys=7. Low glycosylation. It forms disulfide cross-links. Corresponds to some but not all the reticulin fibers of the tissues. It forms very fine fibrils because it is present as procollagen. More abundant in fetal tissues. Decreases with age or in fibrotic processes. Solubilized with pepsin and precipitable from Tris HCl buffers with 1.7 M NaCl.	Most connective tissues where type I collagen is present.	It represents some, but not all, of the reticulin fibers. It is associated with fibronectin and forms the loose connective tissue matrix. It is present in portal triads, terminal venule zones, and centrolobular regions. Its concentration in normal liver is approximately 2 mg/g of fresh tissue and, in cirrhotic liver, it is about 7-8 mg/g of fresh tissue.

Table 1. Collagen Components of Liver Extracellular Matrix —Continued

Collagen Type	Molecular Formula	Special Features	Tissue Distribution	Liver Localization
IV	$\{\alpha 1(IV)\}_3$ $\{\alpha C\}$ $\{\alpha D\}$	Lys+hylys=65; hylys=57. High glycosylation. Low alanine content. 3-hydroxylproline present in relatively large concentrations. Heterogenous. It may contain more than a single chain. Present as procollagen and shows no periodicity. After solubilization with dilute acid, it forms procollagen-size SLS crystallites. Pepsin susceptible site yielding fragments with m.w. of 55 to 180 K. Remains in solution after precipitation of other collagens.	All basement membranes.	It is localized around smooth muscle cells in vessels, around bile ducts, ductules, nerve axons, the endothelial lining of hepatic arteries and lymphatic vessels. In the parenchyma, it is localized around sinusoids between endothelial lining and liver plates. It is associated with laminin in most sites. Its concentration in normal liver is about 0.5 mg/g of fresh tissue. It is increased in cirrhotic liver and occurs in many sites, including in the heavy bundles that surround "regenerative nodules."
V	$\{\alpha A\}_3=\{\alpha 1(V)\}_3$ $\{\alpha B\}_3=\{\alpha 2(V)\}_3$ $\alpha A\{\alpha B\}_2=$ $\{\alpha 1(V)\}\{\alpha 2\}_2$	Collagen B. Lys—hylys—55; hylys—35. High glycosylation. Collagen A, normal hylys—lys; hylys—13. Collagen A has a lower melting temperature than collagen B. Low alanine content. It contains small amounts of 3-hydroxyproline. It might be associated with the exoskeleton of mesenchymal cells. Solubilized with pepsin and precipitable from Tris HCl buffer with 4.0 M NaCl.	Present in most tissues in 3 to 5 percent concentration. Kidney mesangium, liver perisinusoidal structures, intestinal basal lamina, lung.	It is present in portal and terminal venule areas. In the centrolobular region, it shows a perisinusoidal distribution and may be in close contact with the plasma membrane of hepatocytes. In normal liver, its concentration is close to 0.5 mg/g of fresh tissue. Increases in cirrhotic liver and is found in most places mixed with other collagen types.

(From Rojkind, M., and Kershenobich, D. Hepatic fibrosis. *Clinics in Gastroenterology,* 10(3): Sept. 1981. Copyright 1981 by W. B. Saunders Co. [Phila., PA].)

Table 2. Noncollagenous Glycoprotein of Liver Extracellular Matrix

Proteins	Molecular Organization	Special Features	Tissue Distribution	Liver Localization
Fibronectin	2 subunits of 220 K, each joined by a single disulfide cross-link near the carboxyl-terminal end.	It has several domains represented by sequences that bind to cells, collagen, glycosaminoglycans, and glycoprotein.	Widespread distribution	It is co-distributed with type IV collagen. It is present in the perisinusoidal region, perhaps associated with type III collagen. It is absent from the interstitial matrix of portal triads. It is increased in liver cirrhosis, but its concentration is not known.
Laminin	Disulfide cross-linked polymer of 220 K monomeric subunits.	It appears to have an important role in morphogenesis and in cell-cell aggregation.	Co-distributed with type IV collagen in most tissues	Co-distributed with type IV collagen except in the perisinusoidal region. It is increased in cirrhotic liver. Its concentration is not known.

(From Rojkind, M., and Kershenobich, D. Hepatic fibrosis. *Clinics in Gastroenterology,* 10(3): Sept. 1981. Copyright 1981 by W. B. Saunders Co. [Phila., PA].)

Table 3. **Carbohydrate Units of Glycosaminoglycans**

Glycosaminoglycan	Sugar in Repeating Disaccharide Unit	Average number of Disaccharide Units	Position of O-Sulfate Groups	Other Sugars
Hyaluronic acid	GlcUA; GlcN	500-2500		
Chondroitin 4- and 6-sulfate	GlcUA; GalN	60-80	GalN-4-S; GalN-6-S	Gal
Heparin and heparan sulfate	GlcN; GlcUA; IdUA	10-20	GlcN-6-S; IdUA-2-S GlcUA-2-S	Gal
Keratan sulfate	GlcN; Gal	10-20	GlcN-6-S; Gal-6-S	NAN = Fuc Man
Dermatan Sulfate	GalN; IdUA or GlcUA	60-80	GalN-4-S	Gal

(From Roden, L., and Horowitz, M.I. In: *Glycoconjugates*, Vol. II. Horowitz, M. I., and Pigman, W., eds., 1978. Copyright 1978 by Academic Press, Inc. [N.Y.].)

Development of an Assay for Mammalian Hepatic Collagenase and Study of the Effect of Ethanol on Enzyme Activity

Isao Okazaki, Lawrence Feinman,
Katsuya Maruyama, Zev Fainsilber,
Masayuki Nakano, and Charles S. Lieber

Native collagen is largely resistant to all known proteolytic enzymes except collagenase, which is operative at neutral pH under physiologic conditions. The first animal collagenase was found from the culture fluid of tadpole skin (Gross and Lapiere 1962; Nagai et al. 1966). Subsequently, collagenase was found in media culture of many kinds of tissues, such as skin, postpartum rat uterus, bone, human gingiva, cornea, leucocytes, synovium, and in the synovial fluid from patients with rheumatoid arthritis (Harris and Cartwright 1977; Harris and Krane 1974). Mammalian collagenase had not yet been definitively observed in the liver of either animals or humans until Okazaki and Maruyama (1974) reported its presence and an increase of its activity in experimental hepatic fibrosis.

Our first objective was to study the effect of chronic ethanol consumption on mammalian collagenase activity of rat liver. Whole liver homogenate was chosen as the enzyme source with which to compare the collagenolytic activity in normal and pathological liver. In order to develop a suitable assay, we had to overcome these problems: (1) The activity might be anticipated to be low since collagen content of rat liver is meager; (2) Liver contains lysomal collagenolytic or proteolytic enzymes such as cathespin B1 (Burleigh et al. 1974). Even if the activity of these enzymes is poor at neutral pH, they cannot be overlooked if the activity of mammalian collagenase is low. It therefore became very important to eliminate the activity resulting from these other proteinases in liver homogenate. This was accomplished by exploiting specific differentiating characteristics of these enzymes. Neutral collagenolytic activity was measured using reconstituted collagen fibrils as substrate under the following conditions, pH 7.8 at 25°C (7.6 at 35°C) to exclude acid proteinases and addition of P-chloromercuribenzoate (CMB) to inhibit thiol pro-

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NOTE: Figures appear at end of paper.

teinases. The activity remaining in the presence of EDTA, which is due to serine proteinases, was subtracted to calculate mammalian collagenase activity as the metal proteinase (Okazaki et al. 1977).

To verify the presence of the characteristic cleavage products β A (3/4-length fragments of the β chain) due to mammalian collagenase, a solution assay followed by disc electrophoretic analysis of the reaction mixtures was performed. As shown in figure 1, demonstration of the typical cleavage products due to mammalian collagenase in the reaction mixture under these conditions was accomplished (Maruyama et al. 1981).

In order to study the effect of ethanol consumption on hepatic collagenase, this assay was used on the models of liver injury developed by Lieber and DeCarli (1974; Lieber et al. 1974). Twenty baboons were pair-fed for up to 5 years with either a nutritionally adequate control diet or a diet with 50 percent of carbohydrate calories substituted by alcohol. Hepatic collagenase activity was significantly increased at the fatty liver stage, compared to that of control-fed animals (1.98 ± 0.08 units [μg collagen digested/hr/mg liver protein]; mean ± S.E.M. $P<0.001$), and the increase persisted at the stage of fibrosis (2.16 ± 0.07 v. 1.20 ± 0.08 units, $P<0.001$).

Then the collagenase activity in liver biopsies of 28 patients with various stages of alcoholic liver disease—fatty liver, fibrosis, and cirrhosis—was studied. Patients with hepatic fibrosis had significantly higher hepatic collagenase activity than did those with fatty liver (9.12 ± 0.94 [n=14] v. 4.52 ± 0.54 [n=7] units, $P<0.001$). However, as cirrhosis became manifest, hepatic collagenase activity fell from that of the fibrosis stage to values not higher than those of the fatty liver stage—3.92 ± 0.61 [n=7] units, $P<0.001$. This suggests that the development of cirrhosis might be coincident with or favored by a failure of hepatic degradative pathways to keep pace with hepatic collagen synthesis.

It is of interest that, as shown in figure 2, ethanol feeding for 5 weeks increased collagen-bound hydroxyproline content in the liver of rats (Okazaki et al. 1977), but did not induce hepatic fibrosis in rats. However, in this model, the collagenase activity increased (77.7 ± 4.5 dpm/hr/100mg protein in chronic ethanol-fed rats v. 51.7 ± 2.6 dpm/hr/100mg protein in controls; $P<0.01$). The increase in collagenase may have been sufficient to prevent fibrosis.

To clarify the effect of ethanol on collagenase production by cells, monolayer rabbit synovial fibroblasts were cultured and treated with ethanol, iron, or phorbol myristate acetate (PMA), as described elsewhere (Okazaki et al. in press). Ethanol was added daily to confluent cultures in DMEM containing 0.2 percent lactalbumin hydrolysate. Iron, in the form of ferric nitrilotriacetate (FeNTA), was taken up by fibroblasts and resulted in increased production of latent collagenase, as shown in figure 3. Addition of PMA increased collagenase production by cells markedly. On the other hand, addition of 40 mM or 20 mM ethanol did not increase collagenase production, as shown in figure 4. Metabolites of ethanol or hepatocytes injured by ethanol may induce the increased collagenase production by fibroblasts in the livers of chronic ethanol-fed rats (Okazaki et al. in press).

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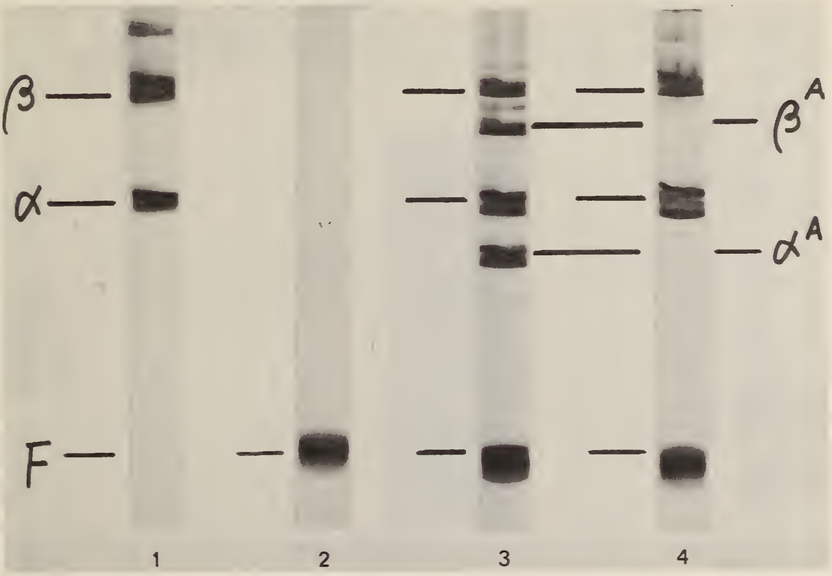
Okazaki, I.; Brinckerhoff, C.E.; Sinclair, J.F.; Sinclair, P.R.; Bonkowsky, H.L.; and Harris, E.D., Jr. Different effect of iron and ethanol on collagenase production by fibroblasts. In: *The Proceeding of Normal and Fibrotic Human Liver*, in press.

Figure 1. Acrylamide Gel Electrophoretic Patterns of Products From Incubations of Collagen With Human Liver Homogenate Demonstrating Collagenase Activity



(1) buffer blank; (2) reaction mixture without collagen substrate; (3) reaction mixture used to assay collagenase activity with P-CMB; (4) reaction mixture used to assay the activity with both P-CMB; and EDTA. α, monomer; β, dimer; F. buffer front. The typical cleavage products of mammalian collagenase action on collagen. αA (3/4-length fragments of α-chain) and βA (3/4-length fragments of β-chain) are demonstrated in No. 3.

Figure 2. Enhancing Effect of Ethanol Feeding for 5 Weeks on Hepatic Collagenase Activity

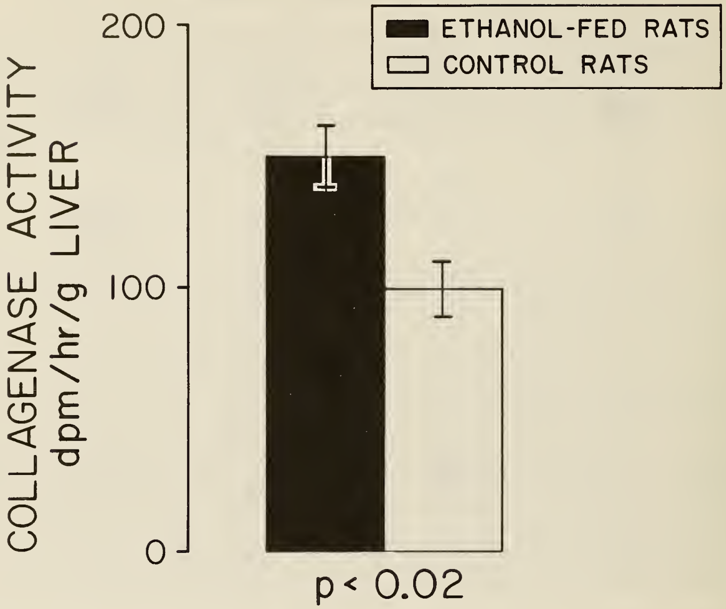
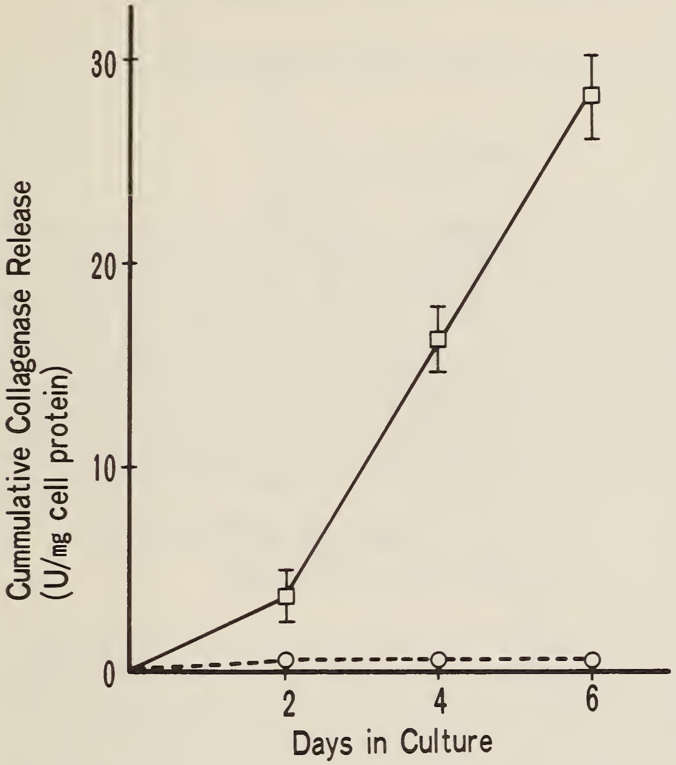
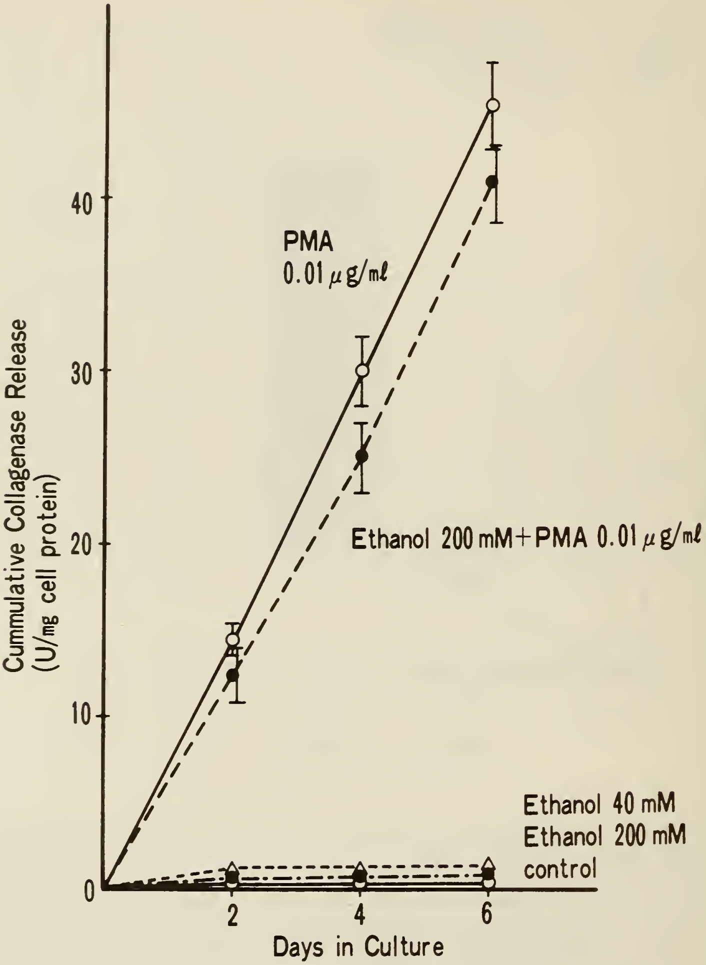


Figure 3. Stimulating Effect of FeNTA on Collagenase Production of Rabbit Synovial Fibroblasts



Cells in 35 mm culture dishes, 2 × 10 cells/dish, in DMEH-LH, were treated with FeNTA (0.5 mM). Medium and FeNTA were replaced every 2 days, and medium was assayed for latent collagenase (m ± S.D.). Cell protein was also determined at the various time points. One unit of collagenase degrades 1 μg collagen fibril/min at 37° C. ☐ FeNTA, O Control. (From Okazaki, I.; Brinkerhoff, C.E.; Sinclair, J.F.; Sinclair, P.R.; Bonkowsky, H.L.; and Harris, E.D., Jr. Iron increases collagenase production by rabbit synovial fibroblasts. *J Lab Clin Med*, 97:396-402, 1981. Copyright 1981 by C.V. Mosby Co. [St. Louis, MO].)

Figure 4. Effect of Ethanol and PMA on Collagenase Production by Rabbit Synovial Fibroblasts



Cells were treated with PMA (0.01 μg/ml) or ethanol (40mM or 200mM). Enzyme production by cells treated with PMA was unaffected by the addition of either 40 mM or 200 mM ethanol.

Serum Procollagen Concentration as a New Tool in the Study of Alcoholic Liver Disease

Eckhart G. Hahn and D. Pencev

Introduction

The development of fibrosis is a hallmark of alcoholic liver disease and escapes biochemical detection. Diagnosis depends on morphologic criteria and may be complicated by sampling errors, contraindications, or lack of cooperation. Clinical parameters to measure hepatic collagen synthesis in the serum are available but not always extensively tested in prospective clinical studies (Hahn and Martini, 1980). The use of a sensitive radioimmunoassay to measure the serum concentration of the amino-terminal procollagen type III peptide in patients with alcoholic liver disease is reported here.

Methods and Patients

Sera of patients selected for a prospective clinical study of chronic liver disease were collected at the time of biopsy. Inclusion of patients in the study required a full clinical, biochemical, and serological evaluation and exclusion of extrahepatic diseases. Laparoscopy was performed when biopsy was inconclusive. Histological evaluation was performed by two pathologists independently and single parameters graded 0-3 (table 1). A detailed description of the radioimmunoassay for the procollagen peptide and clinical procedures is given by Rohde et al. (1979). The normal range of the serum procollagen peptide is 7 ± 5 ng/ml (x ± 2.5 SD).

After 2 years, 56 patients with alcoholic liver disease were available for studies. Two had fatty liver; 19 had fatty liver and fibrosis; 18, alcoholic hepatitis; and 17, alcoholic cirrhosis. Stepwise discriminant analysis was performed with a BMDP 7M program.

Results

In the 2 patients with fatty liver and no other histological changes,

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the serum procollagen was normal (figure 1). In the patients with additional fibrosis, the values ranged from normal in 11 of 19 to 25 ng/ml in 8 of 19. A marked increase of the serum procollagen is observed in all of 18 patients with alcoholic hepatitis and in 15 of 17 with alcoholic cirrhosis. The two cirrhotic patients with normal procollagen values were classified "inactive" by the pathologists and had no signs of portal hypertension (including laparoscopy). The highest value was measured in a patient with alcoholic hepatitis and was 13 times the upper 95-percent confidence limit (160 ng/ml).

Stepwise discriminant analysis of combined laboratory and procollagen values revealed a sensitivity of 0.8 for separation of fatty liver with fibrosis, alcoholic hepatitis, and cirrhosis; specificity was 0.8-0.9. Only the procollagen peptide and prothrombin time were significantly contributing to this separation, procollagen peptides having the highest rank. Fatty liver plus fibrosis and alcoholic hepatitis taken alone were separated with a predictive value (= sensitivity × specificity) of 0.89 by the serum procollagen values alone; no other laboratory parameter contributed significantly. This was surpassed only by the addition of histological data.

Discussion

Hepatic fibroplasia in alcoholic liver disease is an active process involving enhanced collagen synthesis and deposition. Various parameters of collagen synthesis have been used to measure this process in biopsies and serum (Hahn and Martini 1980). Data on sensitivity and specificity are generally not available. In 56 patients with alcoholic liver disease, subgroups were clearly separated by the measurement of the serum concentration of the amino-terminal procollagen type III peptide. The subgroup with low fibroplastic activity (fatty liver plus fibrosis) was separated from the subgroup with high fibroplastic activity (alcoholic hepatitis) by serum procollagen alone. This indicates that the measurement of products of collagen synthesis (procollagen peptides) may be useful in the study and clinical evaluation of alcoholic liver disease.

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Figure 1. Procollagen Peptide in Serum of Patients With Alcoholic Liver Disease

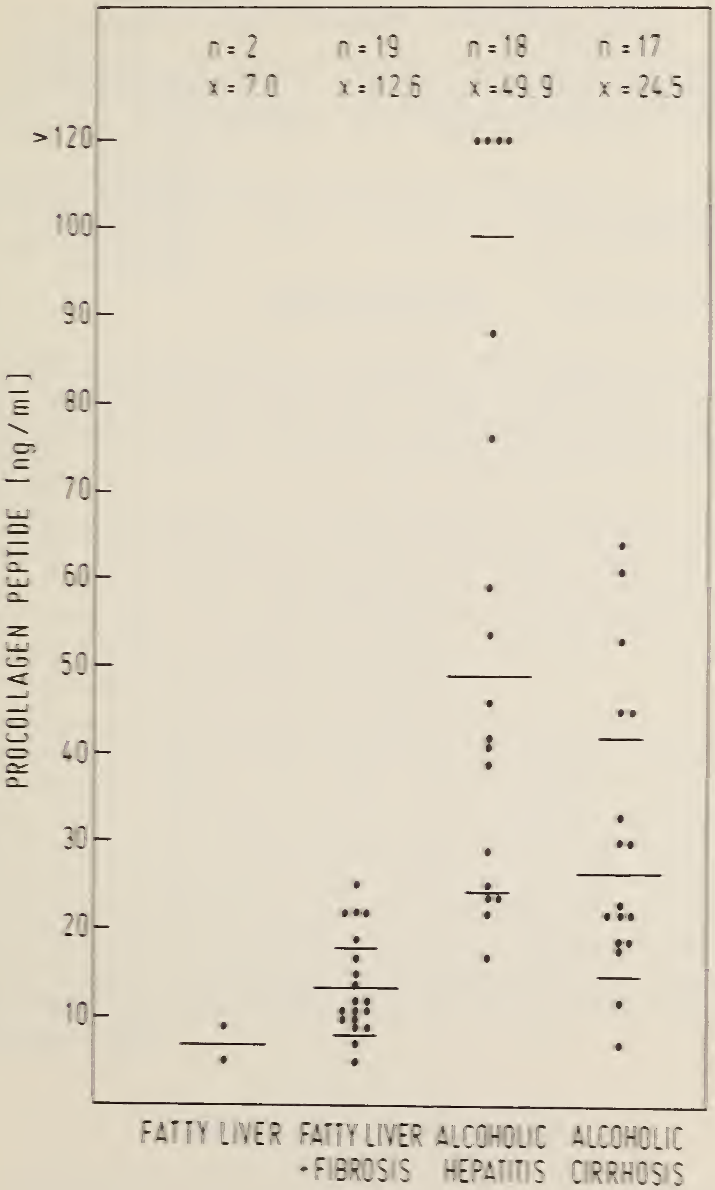

Table 1. Parameters Evaluated and Graded 0–3 in Histologic Analysis of Liver Biopsies

Portal Tract (Septum)	Parenchyma
Portal Enlargement (PE)	Lobular Fibrosis (LF)
Portal Inflammation (PI)	Lobular Necrosis (LN)
Ductular Proliferation (DP)	Mallory Bodies (MB)
	ITO-Cells (IC)
	Kupffer-Cells (KC)
Periportal Region (Septal-Parenchymal Border)	Cholestasis (CO)
	Iron Deposits (FE)
	Steatosis (FA)
Periportal Necrosis (PN)	

Distribution of Hepatic Collagens, Elastin, and Structural Glycoproteins During the Development of Alcoholic Liver Injury in Baboons

Eckart G. Hahn, Rupert Timpl, and Charles S. Lieber

Introduction

In alcoholic hepatic fibrosis, the nature of the fibroplastic cells and their regulation remain obscure. Increased numbers of Ito cells (Bronfenmajor et al. 1966) and myofibroblasts (Bhathal 1972; Rudolph, et al. 1979) were observed in human alcoholic fibrosis or cirrhosis.

Biochemical (Kojima et al. 1975; Rojkind et al. 1979), histochemical (Scheuer 1980), and immunohistological (Biempica 1980; Hahn et al. 1980; Wick et al. 1978) data suggest an increase of all known structural components of connective tissue in alcoholic fibrosis and cirrhosis of the liver, but little information exists about the individual development of this characteristic pattern.

Baboons fed a high-protein liquid diet containing alcohol equivalent to 50 percent of total calories sequentially develop fatty liver, fibrosis, and cirrhosis (Lieber et al. 1975). Certain aspects of this model closely mimic the clinical course and morphologic appearance of human alcoholic liver disease (perivenular sclerosis with early portal hypertension, intralobular fibrosis, formation of septa), and may thus be suitable to study the sequence of events during hepatic connective tissue formation. This paper describes the distribution of hepatic collagens, elastin, and structural glycoproteins during the development of alcoholic liver injury in baboons and correlates it with proliferating fibroplastic cells.

Material and Methods

Alcoholic liver injury was induced in baboons as described by Lieber et al. (1975). Twenty-two baboons were investigated; 11 were pair-fed

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NOTE: Figures appear at end of paper.

controls and had a normal liver as shown by conventional histology. Three had a fatty liver, two had perivenular sclerosis, two had perivenular sclerosis plus perisinusoidal fibrosis, three had already formed septa, and one animal had early cirrhosis. The period of alcohol feeding varied between 5 and 102 months and did not correlate with the stage of the fibrotic lesion. Liver biopsies of these animals (representing the complete sequence of alcoholic fibrosis and the pair-fed controls) were snap-frozen; unfixed, air-dried sections were processed for immunofluorescence or elastin staining. Antibodies to collagen type I, procollagen type I, collagen type III, procollagen type III, collagen type IV, fibronectin, and laminin (Timpl et. al. 1979) were used in this study. All these antibody reagents were affinity purified, extensively tested, and used under standard conditions for indirect immunofluorescence (Timpl et al. 1977). Elastin was identified by orcein staining, using the Elastica-van Gieson modification. For electron microscopy, pieces of the same biopsies as used for indirect immunofluorescence were fixed in glutaraldehyde, post-fixed with osmium tetroxide, dehydrated by alcohol, embedded in Epon 812, and stained with uranyl acetate and lead citrate.

Results

Normal Liver

Procollagens and collagens types I and III, collagen type IV, and fibronectin were deposited along the sinusoidal lining (control-section figure 1A; figure 1B-1E). In the portal tract, type IV collagen and fibronectin were sparse in a punctate pattern, but basement membranes were strongly stained. Small terminal veins had a slender wall composed of types I and III collagen (figure 1F) and an inner basement membrane staining for type IV collagen and fibronectin. Laminin stained exclusively basement membranes and was absent from the perisinusoidal space (figure 1G). Elastin was detected in the wall of small and large arteries, of medium and large portal and hepatic veins, and in small amounts in the interstitial matrix of portal fields.

Fibrosis and Cirrhosis

A marked increase of intensity and area of staining was observed for procollagens and collagens I and III and fibronectin, most markedly around the hepatic venules (figure 2A), in the Disse space and around portal tracts in early stages and along the septal-parenchymal border of cirrhosis. Fibrotic areas also stained for type

IV (basement membrane) collagen (figure 2B) and elastin (figure 2C). Laminin appeared in the Disse space after alcoholic feeding and was most prominent near the septal margins in cirrhosis (figure 2C-D). Some staining also was observed in the fibrotic matrix.

Fibroplastic Cells

By electron microscopy, in both early and late stages of the alcoholic lesion, an increasing number of cells were found. The cells are spindle shaped; the nucleus is lobulated; dense bodies are present along the inner side of the cell membrane; and, most interestingly, a basal lamina is visualized along the outer side of the cell membrane. There is a rich and extended endoplasmic reticulum, and dense collagen fibrils are deposited in juxtaposition to these cells. Thus, they share properties of smooth muscle cells and fibroblasts and fulfill the criteria of myofibroblasts described before in the scar tissue of full-blown human alcoholic cirrhosis (Bhathal 1972; Rudolph et al. 1979). They also share features with fat-storing or Ito cells, and the presence of lipid droplets in the cytoplasm can be demonstrated.

Discussion

These observations reveal close similarities of human (Hahn et al. 1980) and baboon alcoholic liver fibrosis in the major structural components and their distribution. The fibrosis develops through the deposition of collagens I and III and fibronectin around terminal veins and along perisinusoidal spaces, later forming septa. Elastin, type IV collagen, and laminin are present in all fibrotic areas in a punctate pattern. Laminin is detected also along sinusoids, although it is absent in normal liver. In prefibrotic stages, that is, in fatty liver, enhanced perisinusoidal staining for laminin is sometimes observed. The composition of the fibrotic tissue is similar, regardless of lobular location and stage of disease, and suggests the involvement of a cell of wide biosynthetic potential. This cell as visualized by electromicroscopy is distinct from fibroblasts and may be related to the myofibroblasts described in human alcoholic cirrhosis (Bhathal 1972; Rudolph et al. 1979). Indeed, proliferation of myofibroblasts was observed in the alcohol-fed baboons in association with the development of fibrosis. The myofibroblasts could be demonstrated in fibrotic areas in increasing numbers. They were often surrounded by a basal lamina and always by collagen bundles. Frequently, fat droplets were detected within these cells, which suggests a relation of myofibroblasts to Ito cells.

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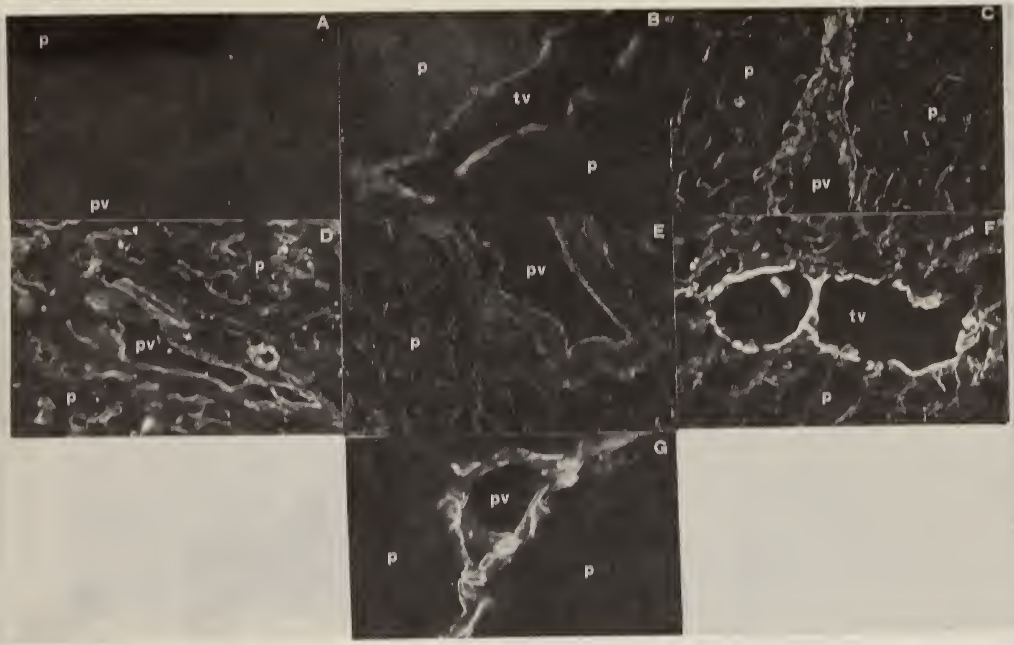
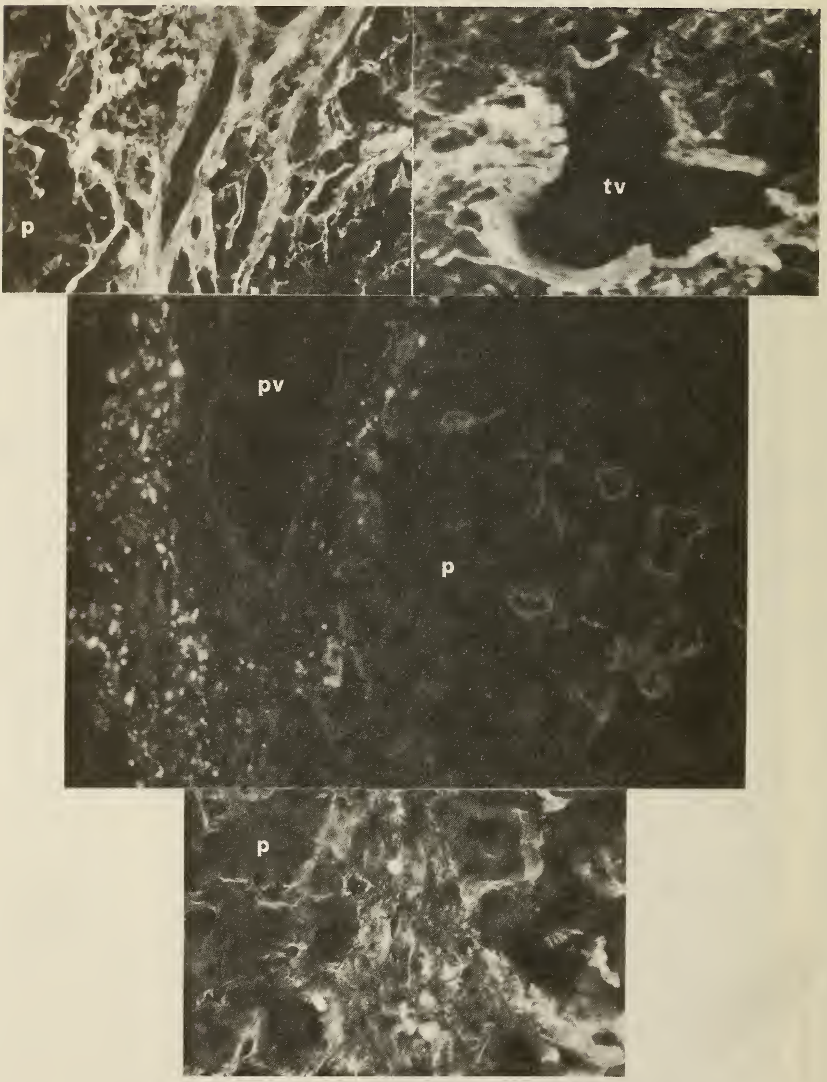
Figures 1A–G. Distribution of Connective Tissue Proteins in Normal Baboon Liver (Staining by Indirect Immunofluorescence)



A *Control Section*, Portal Field, ×300.
B *Anti-type I Collagen*, Weak Staining of Wall of Terminal Vein and Perisinusoidal Spaces, ×480.
C *Anti-Procollagen III*, Staining of Portal Field and Perisinusoidal Spaces, ×300.
D *Anti-Type IV Collagen*, Staining of Basement Membranes of Small Portal Field and of Perisinusoidal Spaces, ×300.
E *Anti-Fibronectin*, Staining of Inner Layer of Portal Vein and Perisinusoidal Spaces, ×300.
F *Anti-Procollagen III*, Staining of Wall of Terminal Vein and Perisinusoidal Spaces, ×300.
G *Anti-Laminin*, Staining of Basement Membranes of Small Portal Field, No Staining of Perisinusoidal Spaces, ×480.
P = Parenchyma; TV = Terminal Vein; PV = Portal Vein

Figures 2A–D. Distribution of Connective Tissue Proteins in Fibrotic (2B) and Cirrhotic Baboon Liver (Staining by Indirect Immunofluorescence)



A *Anti-Procollagen I*, Portal and Pericellular Fibrosis. Intense Staining (Compare Fig. 1B) ×300.

B *Anti-Procollagen I*, Perivenular Sclerosis. (Compare Fig. 1B and 1F) ×300.

C *Anti-Laminin*, Staining of Perisinusoidal Spaces Near Septum. Septal Stroma Negative. Note Autofluorescence of *Elastin* (Verified by Orcein Staining) in Fibrotic Area of Septum ×480.

D *Anti-Type IV*, Staining of Perisinusoidal Spaces and of Fibrotic Area × 480.

P= Parenchyma; TV= Terminal Vein; PV= Portal Vein

Ultrastructure of Perivenular Fibrosis in Alcohol-Fed Baboons*

Masayuki Nakano and Charles S. Lieber

Introduction

The pathogenesis of alcoholic cirrhosis is still a subject of debate. Generally, it is believed that fibrosis develops after tissue necrosis as a part of a cicatrization process (Galambos and Shapire 1973; Galambos et al. 1977). On the other hand, alcoholic liver disease is associated with fibrosis in the centrilobular area, pericellular fibrosis (Popper and Lieber 1980), and perivenular sclerosis (Van Waes and Lieber 1977). These lesions imply an increase of fiber in perisinusoidal and around the terminal hepatic venules without obvious hepatocellular necrosis. Thus, the lesions could be considered as active fibroplasia. However, the cellular origin of fibrosis has not been clear. Recent data demonstrate that perisinusoidal cells (Bartok et al. 1979), fibroblasts (Stenger 1965), or fat-storing cells (Minato et al. 1980; Okanoue et al. 1980; and Ito et al. 1980) play a role in intralobular fibrosis. From our laboratory, the development of perivenular fibrosis in early fatty liver has been reported as an important precursor lesion to alcoholic cirrhosis (VanWaes and Lieber 1977). The purpose of this paper is to clarify which cells contribute to the development of this lesion.

Material and Methods

Twenty-two pairs of baboons were fed ethanol-containing or control liquid diets as described earlier (Lieber and DeCarli 1974). Liver tissue was obtained by surgical or percutaneous needle biopsies at 6- to 12-month intervals, over a 3- to 102-month period. One part of the liver tissue was fixed with 10 percent formalin for paraffin section; the other part was fixed with ice-cold 2.5 percent glutaraldehyde cacodylate buffer (pH 7.4) for at least 2 hours, postfixed with 1 percent cacodylate-buffered osmium tetroxide for 2 hours, dehydrated

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NOTE: Figures and tables appear at end of paper.

* The original studies summarized here were supported by U.S. Public Health Service Grant AA 03508 and the Veterans Administration. The study results are being published in detail in the *American Journal of Pathology*.

with graded alcohol, and embedded in Epon 812. Paraffin sections were stained with Hematoxyline and eosin (H and E) and Masson trichrome for routine examination. For morphometric studies, Epon thick sections (1 μm) were stained with toluidine blue, and terminal hepatic venules were examined for the presence of perivenular fibrosis (previously called perivenular sclerosis). All the terminal hepatic venules were photographed; the size of the diameter, inner circumference, and fibrotic rim of the venule and the number of mesenchymal cells in the fibrotic rim of the venule were measured. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by electron microscope.

Results

Light Microscopic Findings

All alcohol-fed baboons developed fatty liver. Twenty-two baboons were examined by sequential biopsies for 3 to 84 months. Seven baboons developed severe fibrosis and cirrhosis within 3 years. Fourteen baboons showed fatty liver (some of them with moderate fibrosis). Perivenular fibrosis appeared as a lesion at the early stage and was observed in only a few terminal hepatic venules. With progression of the experiment, cases of fibrosis increased in number and intensity. In control baboons, 24 terminal hepatic venules were studied, as shown in table 1. Usually the terminal hepatic venule has only a thin layer of connective tissue and a few mesenchymal cells (figure 1). The thickness of all terminal hepatic venules in controls was less than 8 μm; average thickness of the rim was 4.1±0.4 μm.

In alcohol-fed baboons, 56 terminal hepatic venules were studied; they showed variability in thickness of the rims. In thickened terminal hepatic venules, the rims were irregular, and fibrotic tissue spread into the parenchyma along sinusoids (figure 2). In the connective tissue, many mesenchymal cells were observed (figure 2).

Morphometric Findings

The number of mesenchymal cells was assessed by the number of nuclei per mm of venular inner circumference. Since normal veins had an upper limit in thickness of 8 μm, perivenular fibrosis was defined as venular rims of more than 8 μm in thickness (PVF+). Advanced perivenular fibrosis (PVF++) was defined as a rim of more than 16 μm. The number of mesenchymal cells increased with progression of fibrosis (table 1). In terminal hepatic venules without fibrosis in alcohol-fed animals, the average thickness of the rim was the same as that of controls, but the number of mesenchymal cells was increased. The number of mesenchymal cells continued to increase with progression of fibrosis.

Electron Microscopic Findings

Several types of cells were found in the connective tissue around the terminal hepatic venules. In controls, the most common cell is the myofibroblast (table 2). Myofibroblasts are elongated, spindle-shaped cells, located beneath the endothelial cells in the connective tissue (figure 3). Sometimes they form several layers (figure 4). Myofibroblasts have basal lamina-like structures on part of their surfaces (figure 4) and contain bundles of microfilaments with dense areas in the periphery of the cytoplasm (figure 5). They contain many pinocytic vesicles close to the cell membrane, well-developed rough endoplasmic reticulum, and Golgi complexes (figure 5), small mitochondria, and a moderate number of free ribosomes. Nuclei are oval and sometimes irregularly indented. Centrioles were observed relatively frequently, and cilia were seen in some myofibroblasts. Many myofibroblasts contain small fat droplets in their cytoplasm. A synapse-like structure (a myofibroblast-neural junction) was observed in the fibrotic rim of a terminal hepatic venule. Some fibroblasts and Ito cells and a few mononuclear cells also were observed around terminal hepatic venules in the control baboons. Usually, Ito cells were observed at the site of the junction of the sinusoid and terminal hepatic venule. They contain fat droplets, rough endoplasmic reticulum, and well-developed Golgi complexes. Sometimes centrioles were observed. Pinocytotic vesicles were not prominent, and bundles of microfilaments with dense areas or basal lamina-like structures were not observed.

After alcohol treatment, myofibroblasts, fibroblasts, and mononuclear cells increased in number, but the myofibroblasts remained the most abundant mesenchymal cell. Around the myofibroblasts, prominent intercellular fibrils of three different sizes were seen (figures 6 and 7). The finest fibrils, 2-5nm in diameter, formed basal lamina-like layers close to the surface of the cells. The intermediate-sized fibrils, 10-15 nm in diameter, formed bundles; sometimes, these were mixed with the finest fibers. The largest fibrils, with diameters of 50 nm or more, were striated and arranged randomly in the early stage; they formed large compact bundles in later stages. In advanced perivenular fibrosis bonds, there were tree-like branched myofibroblasts among collagen bundles (figure 8). They had many fat droplets and thus resembled Ito cells, but they also had some microfilaments with dense areas and basal lamina-like structures on some parts of their cell surface.

Discussion

This study revealed that, in control baboons, the most common mesenchymal cell around the terminal hepatic venule is the myofibroblast. After alcohol treatment, myofibroblasts, fibroblasts, and mononuclear cells increased in number; myofibroblasts remained the most abundant mesenchymal cell. In early fibrosis, fibrils of various diameters were observed among mesenchymal cells. Close to the surface of the myofibroblasts were prominent microfibrils, and intermediate-sized fibrils were noted. In advanced fibrosis, myofibroblasts were surrounded by collagen fibers of larger diameters. These findings suggest that myofibroblasts produce some types of collagen fibrils, consistent with other findings (Hahn, Timpl, Nakano, and Lieber 1980; Hahn, Wick, Pencev, and Timpl 1980), which found that type I, type III, and type IV collagen are terminal hepatic venules. It is widely believed that alcoholic hepatitis is a precursor lesion of cirrhosis (Galambos and Shapire 1973). However, in our baboon experiment, perivenular fibrosis occurred at the fatty liver stage—without obvious hepatocellular necrosis or infiltration of neutrophils. In hepatic fibrogenesis, perisinusoidal cells (Bartok 1979) have been considered to play an important role (McGee and Patrick 1972). Some of the perisinusoidal cells are so-called Ito (fat-storing) cells (Ito 1951; Ito and Shibasaki 1968), but some investigators used other terms (McGee and Patrick 1972; Schanack et al. 1967; Stenger 1965). Transformation from Ito cell to fibroblast has been postulated(Kent et al. 1976).

In our series of baboons, we observed primary myofibroblasts in the perisinusoidal space, and proliferation of myofibroblasts constituted initial lesion in alcoholic liver fibrosis. Myofibroblasts have been recognized in the granulation tissue of the skin by Gabbiani et al. (1971) and in various other organs (Guldner et al. 1972; Kobayashi 1978) and pathological lesions (Baur et al. 1978; Gabbiani et al. 1972, 1976; Ghadially et al. 1980; Goellner and Soule 1980; Hassan and Olaizola 1979; Hruban et al. 1974; James and Odom 1980; Ohtani and Sasano 1980; Rothouse et al. 1980; Rudolph et al. 1977; Ryan et al. 1974; Salamon and Hamori 1980; Squier and Kremenak 1980; Thomas et al., 1963; Vasudev 1978). It is, therefore, not surprising that myofibroblasts have been observed in liver cirrhosis, which is a kind of scar lesion of the liver (Bhathal 1972; Irle et al. 1974, 1980; Rudolph et al. 1979). Our results revealed the presence of myofibroblasts around the terminal hepatic venules and in the perisinusoidal space, even in normal liver. It can be postulated that myofibroblasts possess contractility like that of smooth muscle cells. Proliferation of myofibroblasts around the terminal hepatic venule could contribute to the portal hypertension that occurs at the stage of fatty liver in alcoholic liver disease (Reynolds et al. 1969) and also in the baboon (Lieber et al. 1966). The synapse-like structure observed between an end of nerve fiber and a myofibroblast suggests that the latter may participate in the control of the hepatic blood flow. There was good correlation between the increase of collagen fibers and the proliferation of myofibroblasts. Morphologically, Ito cells and myofibroblasts have some common characteristics in that both cells seemed to have the ability to produce collagens (Gabbiani et al. 1976; Kent et al. 1976). Fibroblasts, myofibroblasts, and Ito cells may belong to the same cell family, but this has not been established as yet.

In conclusion, our results revealed the presence of myofibroblasts around terminal hepatic venules in normal liver; after alcohol treatment, myofibroblasts increased in number and showed good correlation with the increase of collagen fibers. Proliferation of myofibroblasts around the terminal hepatic venule appeared to be the first detectable lesion in alcoholic fibrosis.

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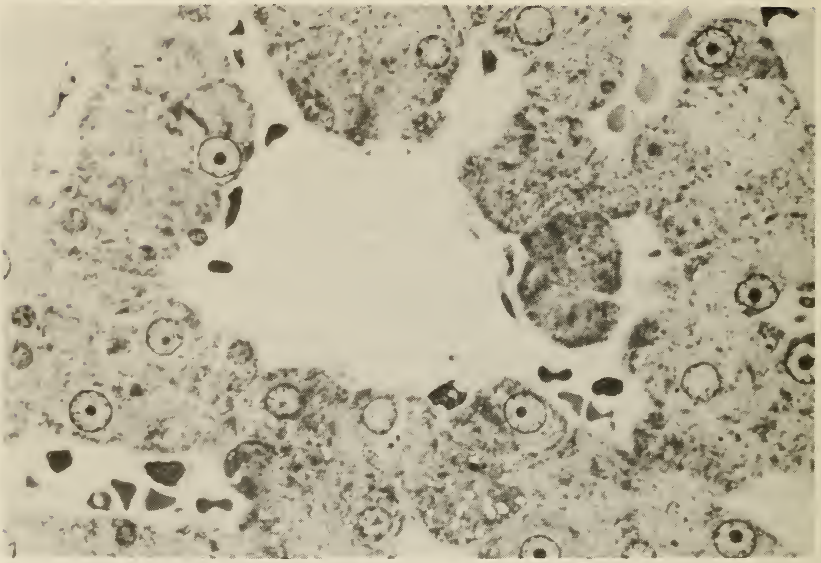
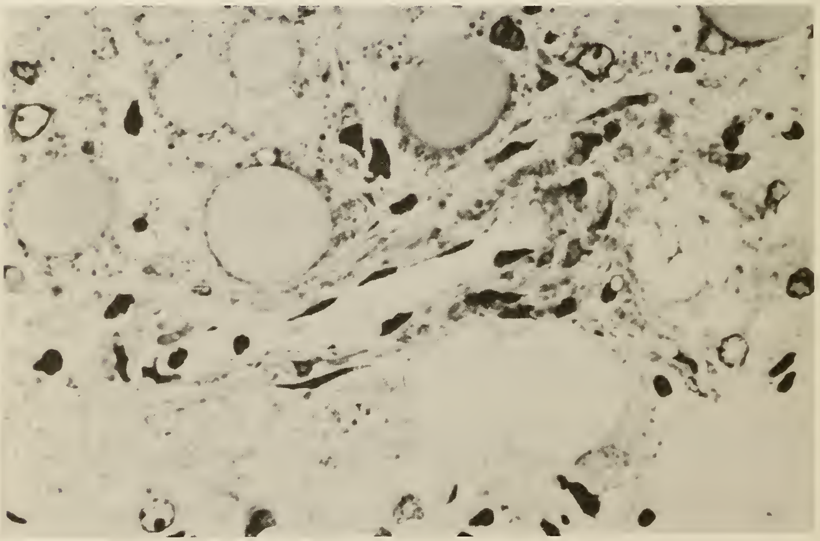
Figure 1. Terminal Hepatic Venule in a Control Baboon



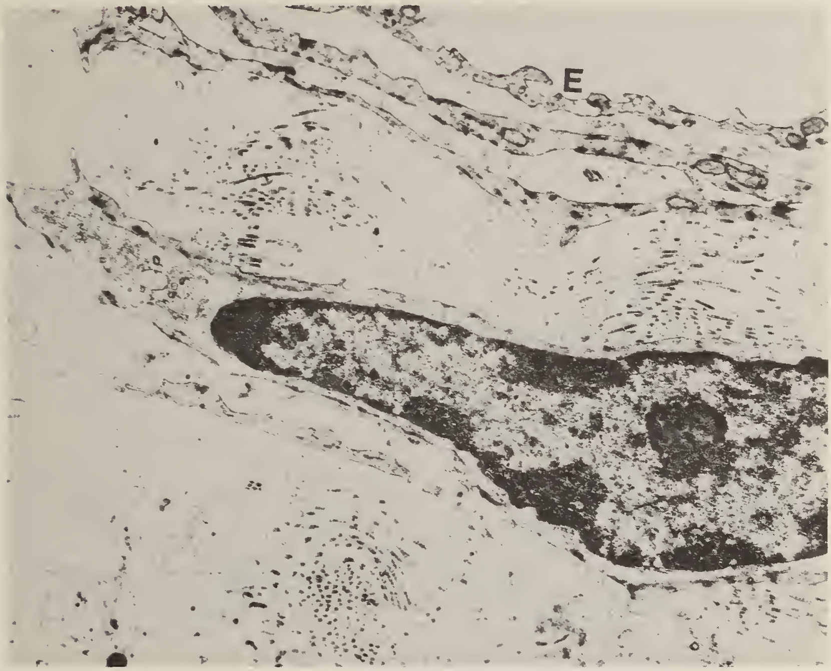
A thin fibrostic rim contains a few mesenchymal cells. (Epon thick section; toluidine blue, X375)

Figure 2. Perivenular Fibrosis in a Baboon Fed Alcohol for 22 Months



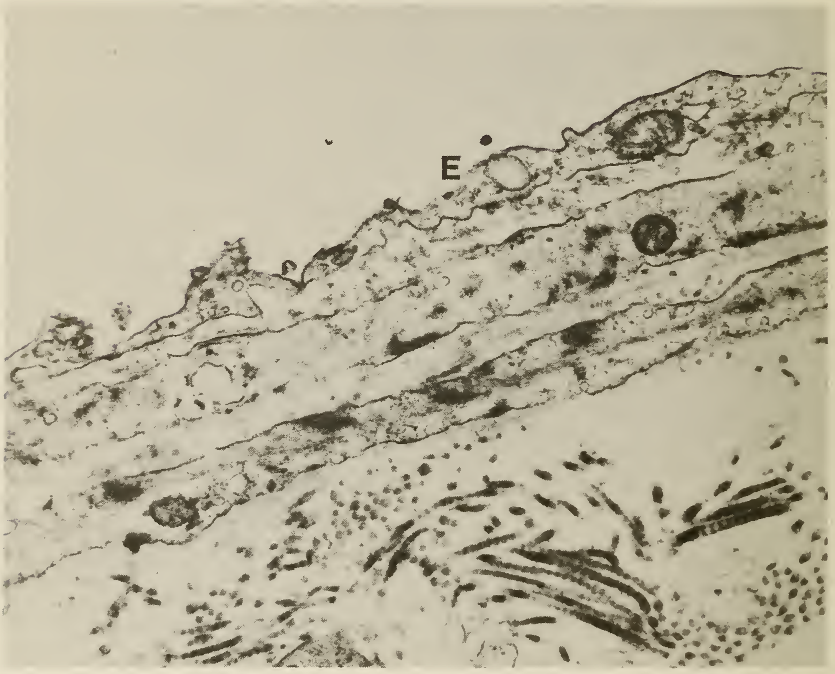
The fibrotic rim is increased in thickness and extends into the parenchyma. There are many mesenchymal cells in the fibrotic rim, some containing fat droplets. Parenchymal cells show fatty changes. (Epon thick section; toluidine blue, X375)

Figure 3. Cells with Elongated Cell Processes Beneath Endothelial Cell (E) in the Wall of the Terminal Hepatic Venule



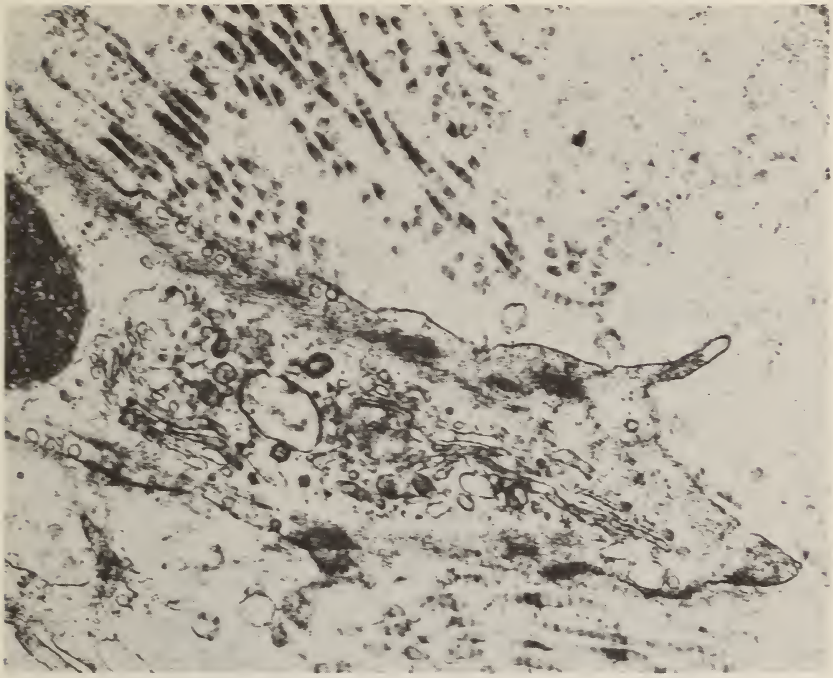
These cells have characteristic structures of myofibroblasts: Microfilaments with dense areas, many pinocytotic vesicles at the periphery of the cells, and basal lamina-like structures on some part of the cell surface. (Uranyl acetate and lead citrate; X9,000)

Figure 4. Higher Magnification of the Wall of the Terminal Hepatic Venule



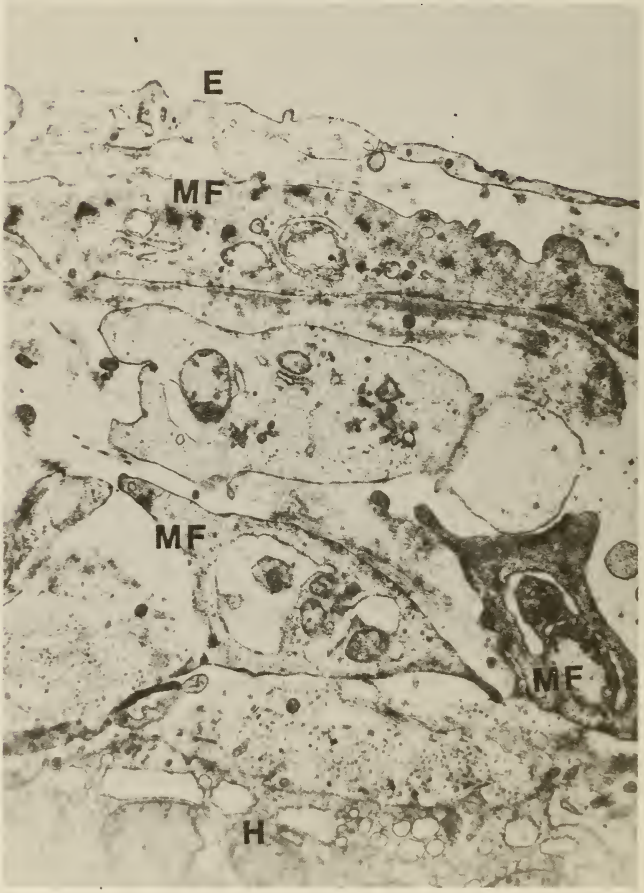
There are two layers of cell processes beneath the endothelial cells (E). They contain microfilaments with dense areas and prominent pinocytotic vesicles; close to the cell surface, there are basal lamina-like structures. (Uranyl acetate and lead citrate; X26,000)

Figure 5. Higher Magnification of Part of Figure 3



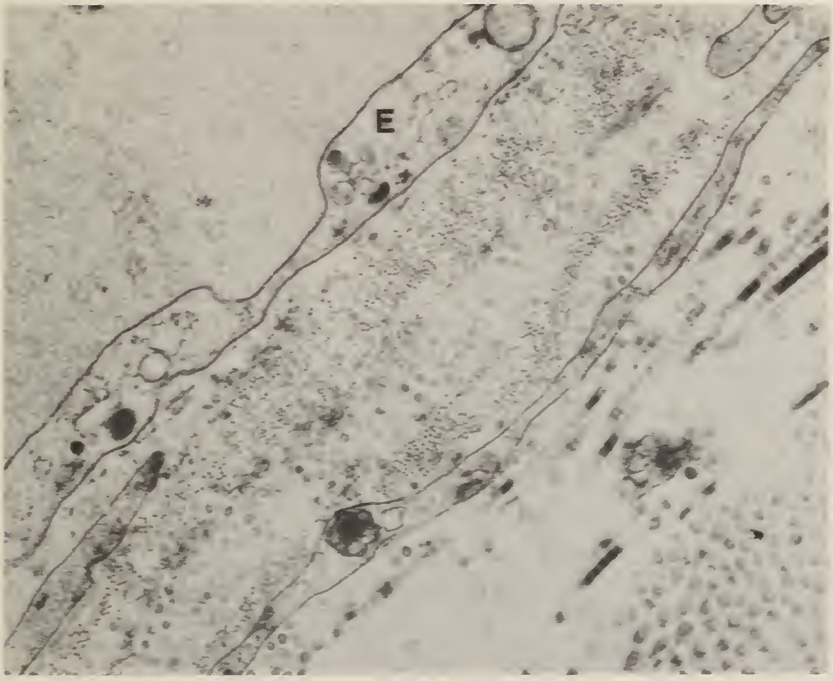
There are bundles of microfilaments with dense areas at the periphery of the cytoplasm and pinocytic vesicles connecting to the cell membrane. The Golgi complex is well developed and there are some coated vesicles near the nucleus. (Uranyl acetate and lead citrate; X25,500)

Figure 6. Terminal Hepatic Venule in an Alcohol-Fed Baboon (15 Months)



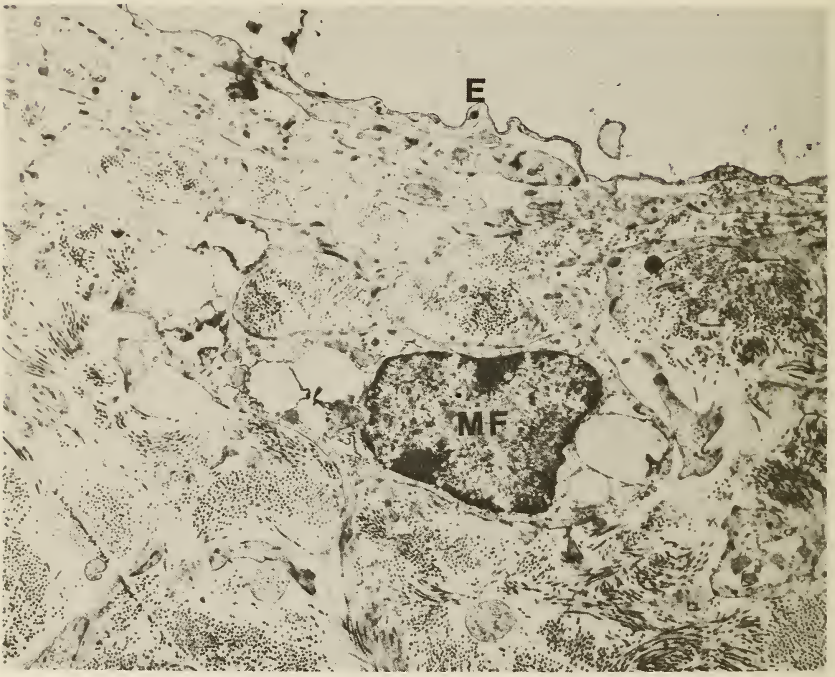
There are many cell processes of myofibroblasts (MF) between endothelial cell (E) and hepatocyte (H). There are many fibrils with diameters of various sizes between cell processes containing dilated, rough endoplasmic reticulum; larger fibrils are observed close to the hepatocyte (H). (Uranyl acetate and lead citrate; X10,400)

Figure 7. **Higher Magnification of the Terminal Hepatic Venule in Advanced Fibrosis**



Three different types of collagen fibrils are recognizable beneath the endothelial cell (E). The finest ones are close to the surface of the cell and form a basal lamina (left lower corner). Most of the intermediate-sized fibrils (with diameters of 10 to 15 nm) appear in cross section as dark points in this picture. The largest fibrils, with a diameter of 50 nm or more, are striated. (Uranyl acetate and lead citrate; X25,000)

Figure 8. Lower Magnification of the Terminal Hepatic Venule in Advanced Fibrosis



There is a tree-like branched myofibroblast (MF), containing fat droplets among collagen bundles. There are many cell processes of myofibroblasts showing dark dense areas of microfilaments and basal lamina-like structures beneath the endothelial cell (E). (Uranyl acetate and lead citrate; X6,000)

Table 1. Quantitative Assessment of Perivenular Fibrosis (PVF) (Mean ± SEM)

Baboons	PVS	Number of THV studied	Diameter of THV(μm)	Thickness of rim(μm)	Number* of cells	P**
Control	−	24	58.2 ± 4.5	4.1 ± 0.4	9.3 ± 0.9	
						<0.05
Alcohol-fed	−	35	46.4 ± 4.2	4.1 ± 0.3	12.5 ± 1.1	
						<0.01
	+	13	54.7 ± 10.1	10.4 ± 0.6	19.2 ± 2.4	
						<0.005
	++	8	53.4 ± 12.0	26.3 ± 2.5	44.0 ± 7.7	

Note: After treatment with alcohol, mesenchymal cells increased in number initially, without thickening of rim. They continue to increase in number, with progressive thickening of rim.

*Mesenchymal cells per mm of inner circumference of the terminal hepatic venule (THV).

**Pertains to number of cells of adjacent group.

Table 2. Cellular Population around Terminal Hepatic Venules (expressed per 100 cells)

PVS	Baboon	MF	Ito	FB	Mono
PVS (−)	control	76	9.5	9.5	5
PVS (−)	alcohol-fed	64	5.5	13.5	17
PVS (+)	alcohol-fed	68	5	15	12
PVS (++)	alcohol-fed	67	1	14	18

MF = Myofibroblasts.
Ito = ITO (fat-storing) cells.
FB = Fibrolasts.
Mono = Other mononuclear cells.
In all groups, the most common cell type was myofibroblast.

Alcoholic Hyalin and the Endoskeleton of the Hepatocyte*

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Nancy C. Benson, and Lynn H. LeFevre

Introduction

The study of isolated alcoholic hyalin (AH) filament ultrastructural and solubility characteristics led to the suggestion that AH represented an abnormal accumulation of intermediate filaments (IF) in affected hepatocytes (French and Davies 1975*a*). Intermediate filaments are normally dispersed in the hepatocyte cytoplasm (Denk, Francke, and Eckerstorfer et al. 1979; Franke, Schmid et al. 1979; French and Davies 1975*a*), but appear in closely packed fascicles about the canaliculus, the nucleus, the centrioles, and traversing the cell from the sinusoid to the canaliculus (Fiskum et al. 1980; Franke, Schmid et al. 1979; French and Davies 1975*b*; Yokota and Fahimi 1979). Thus, it is postulated that when AH forms in an affected hepatocyte it forms at the expense of the normal endoskeleton (IF) system, leaving the hepatocyte without a normal cell shape or organization of organelles (French and Davies 1975*a*; French, Sim, Franke et al. 1977; Huang and Kimoff 1980).

The pathogenesis of this alteration in the hepatocyte endoskeleton is unknown, but several theories have been offered. The first is that the loss of microtubule function is the primary event and the assembly of IFs to form AH is secondary (Denk and Eckenstorfer 1977; French and Davies 1975*a*; French, Sim, Franke et al. 1977). The second theory is that AH filaments are composed of prekeratin, similar to that found in squamous epithelium, and form as a consequence of vitamin A deficiency (Denk, Franke, Kerjascki et al. 1979). A third theory, which is highly speculative but worthy of some consideration, is the idea that AH forms, at least in animals fed carcinogens, as the result of a putative preneoplastic change (Borenfreund et al. 1979; Borenfreund, Higgins, and Carra 1980; Borenfreund, Higgins, and Peterson 1980; French and Burbige 1979; Ihrig et al. 1980).

There is morphologic, biochemical, and immunologic evidence that AH filaments are composed of proteins derived from intermediate filaments (Denk, Franke, Kerjascki et al. 1979; Franke, Schmid et al.

NOTE: Figures and table appear at end of paper.

* This work was supported by the Veterans Administration.

1979; French and Davies 1975*a*). Perhaps the most compelling evidence is immunologic, where antibodies to intermediate filament proteins and human AH hyalin cross-react with IF and AH from human and animal liver cells and from hepatomas (Denk, Franke, Eckerstorfer et al. 1979; Denk, Franke, Kerjascki et al. 1979; Franke et al. 1978; Franke, Denk et al. 1979; French, Sim, and Caldwell 1977; Virtanen et al. 1979).

The present paper reports the result of investigations of the nature of the AH filaments and their relation to the intermediate filament component of the endoskeleton.

Methods

Polypeptide Composition After Deoxycholate Treatment

Both IF and AH filaments resist detergent treatment, whereas microfilaments and microtubules, the two other components of the endoskeleton, disappear after treatment with Triton X-100 (Lehto et al. 1978). In glutaraldehyde-fixed liver, microtubules were removed by deoxycholate and not by Triton X-100 extraction (Yokota and Fahimi 1979). From prior studies, it is known that AH filaments resist extraction by 5-percent deoxycholate (French et al. 1972; Wiggers et al. 1973). This study, therefore, compared the ultrastructural and polypeptide composition of isolated human AH before and after extraction with deoxycholate. AH was isolated from alcoholic hepatitis livers using the Ficoll method of Tinberg et al. (1978). For some experiments, the Ficoll step was omitted to determine whether the Ficoll step improved the purity of the AH fraction. The isolated AH pellet was divided into two parts; one part was resuspended in 5-percent deoxycholate and incubated in a shaking waterbath at 37°C overnight. Deoxycholate was removed by centrifugation at 10,000 rpm for 10 min followed by washing 3 times in isolation medium (IM) without sucrose, with a final suspension in Im without sucrose. The AH pellet not incubated in deoxycholate was similarly washed. The final pellet derived from 40 g starting liver was 1.77 mg protein. Aliquots of the untreated and deoxycholate-extracted pellets were taken for electron microscopic examination and for gel electrophoresis.

Gel electrophoresis was accomplished as reported previously (Sim et al. 1978) using the Laemmli (1970) technique. A 10-percent SDS acrylamide separating gel with 5-percent stacking gel was used. The gel was run in Tris-glycine electrode buffer, pH 8.3, for 1.5 hours, at a constant current of 50 mamp. AH isolates and standards were coelectrophoresced in 1.5 mm slab gels as follows: (1) Molecular weight standard mix 10 μl (Trypsin MW 24,000; ovalbumin MW 43,000; and bovine serum albumin 68,000); (2) Deoxycholate-extracted AH pellet 102 μg protein; (3) Ficoll pellet 100 μg protein. Gels were stained by the Coomassie blue method.

To determine whether protease inhibitors would inhibit degradation of the AH proteins during the isolation procedure, the protease inhibitor phenylmethyl sulfonyl fluoride (0.5 mM) and the protease substrate TAME (para tosyl-L-argine methyl ester, 0.5 mg/ml) were added to the medium in which AH was isolated. Pre-Ficoll pellets, which were isolated using protein inhibitor (5.0 mg AH/40 g liver), and pellets that were isolated without protein inhibitor added (6.5 mg AH/40 g liver) were solubilized in the solvent cocktail and co-electrophoresced as follows: (1) Molecular weight standard mix; (2) Ficoll AH pellet isolated in protease inhibitor (67 μg protein); (3) same as #2, except pre-Ficoll (100 μg protein); (4) pre-Ficoll AH isolated without protein inhibitor (100 μg); and (5) molecular weight standard mix.

Reconstruction of AH Filaments

AH isolates were prepared to the pre-Ficoll stage and incubated overnight at 37°C in 5-percent deoxycholate, and the resultant pellet washed in deionized water (final yield, 7.4 mg protein). The pellet was suspended in a 1-ml solution of 2 M guanidine, 0.05 M mercaptoethanol, and 0.5 M sodium phosphate buffer (pH 7.5), and left stirring overnight at room temperature. The suspension was centrifuged at 12,000 rpm for 5 min. The pellet was washed with 2 ml of solvent by stirring 8 hours, microfuged, and the two supernatants combined. The pellet was repeatedly washed as before, until a total of 10 ml supernatant was obtained. The combined supernatant was filtered overnight. The resulting clear filtrate was dialyzed over the weekend against deionized water, and again overnight against fresh water. The precipitate was centrifuged and fixed for 2 hours in 2.5-percent glutaraldehyde in sodium cacodylate buffer, and postfixed in 1 percent O_5 O_4 and en bloc stained with uranyl acetate, dehydrated, and embedded in Spurr's. Thin sections were cut and viewed in an electron microscope.

Positive Staining and Platinum Coating of AH Filaments on Formvar Coated Grids

AH filaments were isolated by preparing a pre-Ficoll pellet and extracting the pellet by an overnight incubation in 5-percent deoxycholate at 37°C. The resulting suspension was washed 3 times in a microfuge tube with isolation medium without sucrose. The final suspension was sonicated and a drop of the material was immediately placed on Formvar coated grids. After 4 min, the drops were absorbed with filter paper. A drop of 2-percent aqueous uranyl acetate (pH 4.8) was placed on each grid for 2 to 4 min, then washed with distilled water, dried, and viewed with an electron microscope. Similarly prepared grids were placed in an evaporator and platinum coated at 50 V for 5 sec while the stage was rotating 1×10^{-5} Torr.

Three-Dimensional Stereopairs of AH Filaments

AH filaments were isolated by the method of French et al. (1972). The filament staining was enhanced by fixation with a solution containing ruthenium red after the method of Luft (1971). Sections of plastic-embedded material (200-400 nm) were cut and viewed at an accelerating voltage of 100 kV with an electron microscope equipped with a goniometer stage. Negatives for stereopairs were exposed at angles of +6 and −6°.

Results

Electrophoresis

AH filament isolates obtained for gel electrophoretic separation of protein bands showed the same highly reproducible pattern (figures 1 and 2). The mobilities of polypeptides derived from five separate isolates yielded five separate dominant bands. Band 1 had a calculated molecular weight of 56,469 ± 177 (SEM) daltons. The second was 50,210 ± 841 daltons. This band sometimes separated into a doublet of 51,583 ± 297 and 50,004 ± 116 daltons. The third band was 46,669 ± 262 daltons. The fourth was 43,412 ± 150 daltons and the fifth was 30,287 ± 692 daltons.

AH extracted by deoxycholate treatment was decidedly cleaner than unextracted material as accessed by gel electrophoresis (figure 1). Pre- and post-Ficoll pellets did not differ by electrophoresis, suggesting that little is achieved by this preparatory step in terms of removing contaminating proteins. Electron micrographs of AH filaments before and after extraction by deoxycholate (figures 3 and 4) correlate with the electrophoresis results in that the filaments were decidedly easier to delineate because of the loss of background material after deoxycholate treatment.

Gel electrophoretograms of AH filaments prepared in a medium containing a protease inhibitor did not differ from those prepared in the usual isolation medium (figure 2). This suggests that protease inhibitors are not necessary in the isolation medium to prevent proteolysis of AH filament proteins.

Reconstitution of Filaments

Electron micrographs of pellets derived from dialyzed, solubilized AH isolates showed aggregates of filaments with an arrangement that closely resembled the presolubilized AH filaments. The reconstituted filaments were in small discrete clusters, suggesting that they formed by growth from a single filament. The filaments were straight and branched and had diameters similar to those of AH filaments (figure 5). Independent aggregates of thin filaments, possibly actin microfilaments, were also occasionally encountered.

Positive Staining and Platinum Coating of AH Filaments

AH filaments dispersed by sonication of AH isolates were seen in small clusters when subjected to positive staining and platinum coating. The filaments were straight, but had irregular outlines with central, nonstaining cores (figures 6 and 7). Branching of filaments was clearly seen (figures 6, 7, and 8).

Three-Dimensional Stereopairs of AH Filaments

When AH filaments were viewed on stereopaired photographs (to see the filaments in three dimensions), branching filaments were identified (figure 9). The branches were not easily located because of superimposition of filaments; sometimes filaments lay side by side, simulating branching. Tubularity was very easy to see in three dimensions. Connections or adherence of filaments to vesicles was also seen easily in three dimensions (figure 8).

Discussion

The characteristics of isolated and reconstituted AH filaments are consistent with their origin from IF proteins. AH filaments were found to be straight, branching, tubular filaments, which form apparent connections with vesicular membranes similar to the IFs seen in a variety of cells (Benson et al. 1980; Denk, Franke, Eckerstorfer et al. 1979; Denk, Franke, Kerjascki et al. 1979; Fiskum et al. 1980; Franke et al. 1978; Franke, Schmid et al. 1979; French 1980; French and Davies 1975*b*; Lehto et al. 1978).

These observations differ from the conclusions that AH filaments were nonbranching (Denk, Franke, Eckerstorfer et al. 1980; Denk, Franke, Kerjascki et al. 1980). Solubility characteristics of AH filaments are also quite similar to normal cellular IF in that they resist solubilization by detergents such as Triton X-100 and deoxycholate (French et al. 1972; Heuser and Kirschner 1980; Lehto et al. 1978; Wiggers et al. 1973; Yokota and Fahimi 1979). Negative staining and reconstitution of the AH filaments help substantiate branching and morphologic similarity to reconstituted intermediate filaments from other cell types (Fellini et al. 1978; Zackeroff and Goldman 1979).

The polypeptide composition of the AH filaments resembles that of IFs of different types (Lazarides 1980), but the combination of polypeptides does not fit exactly the pattern of any one type of filament. It best resembles the prekeratin type of filaments, which contain polypeptides with molecular weights of 63, 60, 58, 52, and 51 × 10^3. (See table 1). This is in agreement with the immunofluorescent studies of Denk, Franke, Eckerstorfer et al. (1979); Denk, Franke, Kerjascki et al. (1979); Franke et al. (1978); Franke, Denk et al. (1979); and Franke, Schmid et al. (1979). The pattern of human AH filament polypeptide bands, i.e., 56,000; 50,000; 47,000; 43,000; and 30,000, bears some resemblance to (but also differs from) the pattern of mouse AH polypeptides (Franke, Denk et al. 1979). This is the first time these authors have found a polypeptide band of 43,000 daltons. This band could be actin and is similar to other study results (Borenfreund, Higgins, and Peterson 1980). Finding this band could be related to the fact that the AH isolates in the present studies were predominantly type II, in contrast to prior studies where the isolates were predomi-nantly type III (Tinberg et al. 1978). Some investigators have found that actin persisted in IF fractions after actin extraction (Borenfreund, Higgins, and Peterson 1980; Hubbard and Lazarides 1979; Sim et al. 1978). In the present study, actin-like microfilament aggregates were seen in addition to intermediate filament-like aggregates when solubilized AH isolates were reconstituted by dialysis.

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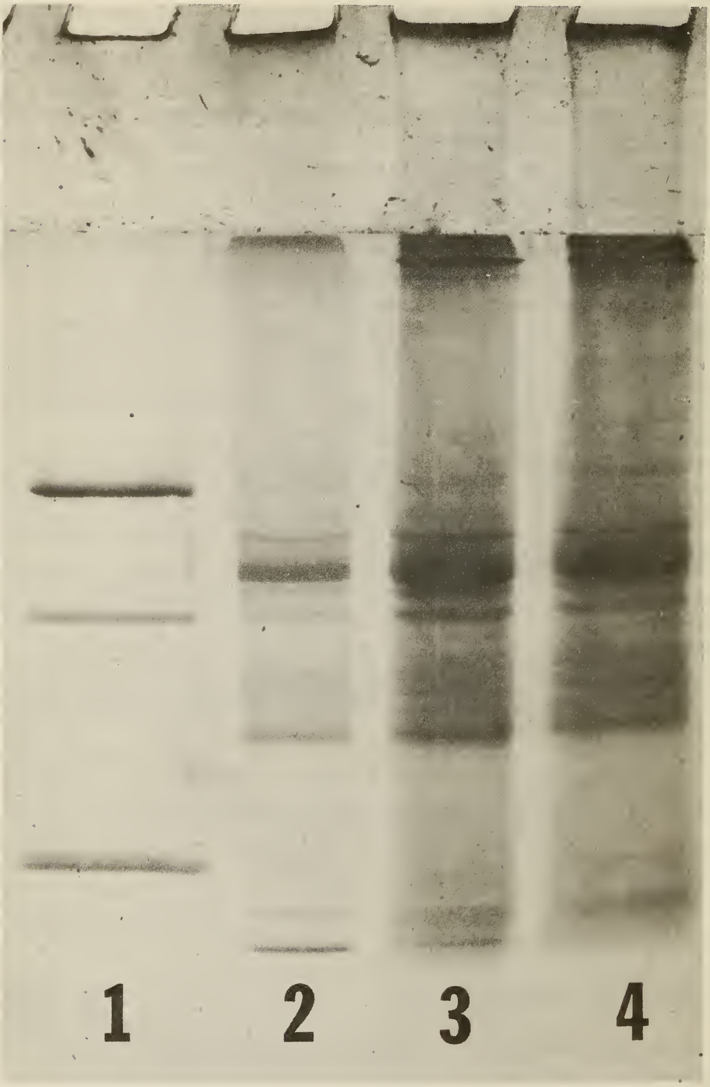
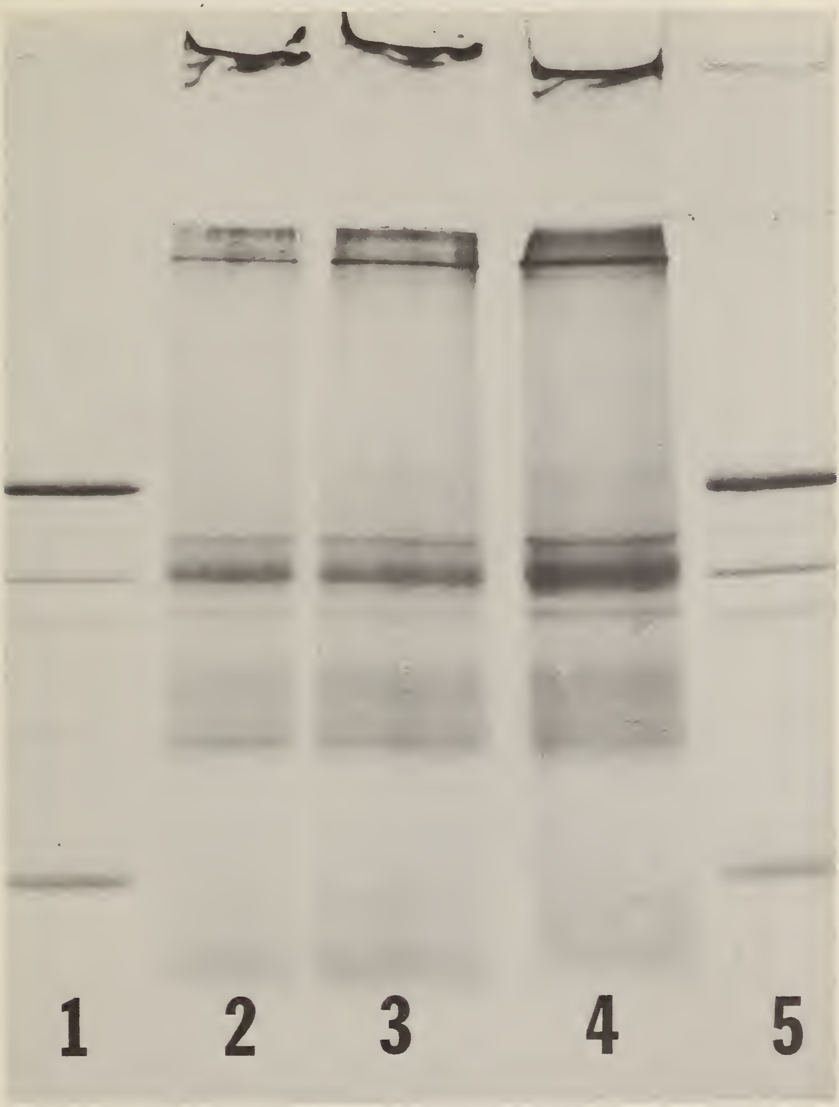
Figure 1. Slab-Gel Electrophoresis of Alcoholic Hyalin Isolates



1. Molecular weight standard.
2. Deoxycholate-extracted alcoholic hyalin pellet
3. Ficoll pellet of alcoholic hyalin
4. Crude pre-Ficoll pellet of alcoholic hyalin

Note: Pre-Ficoll and post-Ficoll pellets do not differ (3 and 4), but deoxycholate extraction reduced background staining, eliminates the high molecular weight protein band, and increases the low molecular weight protein fragments at the solvent front.

Figure 2. Slab-Gel Electrophoresis of Alcoholic Hyalin Isolates



1. Molecular weight standard mix.
2. Post-Ficoll alcoholic hyalin pellet isolated in medium containing protease inhibitor.
3. Same as 2, except pre-Ficoll.
4. Pre-Ficoll pellet isolated in medium that did not contain protease inhibitor.

Note: 2, 3, and 4 do not differ from each other.

Figure 3. Electron Micrograph of AH Filaments Before Deoxycholate Extraction (X62,400)

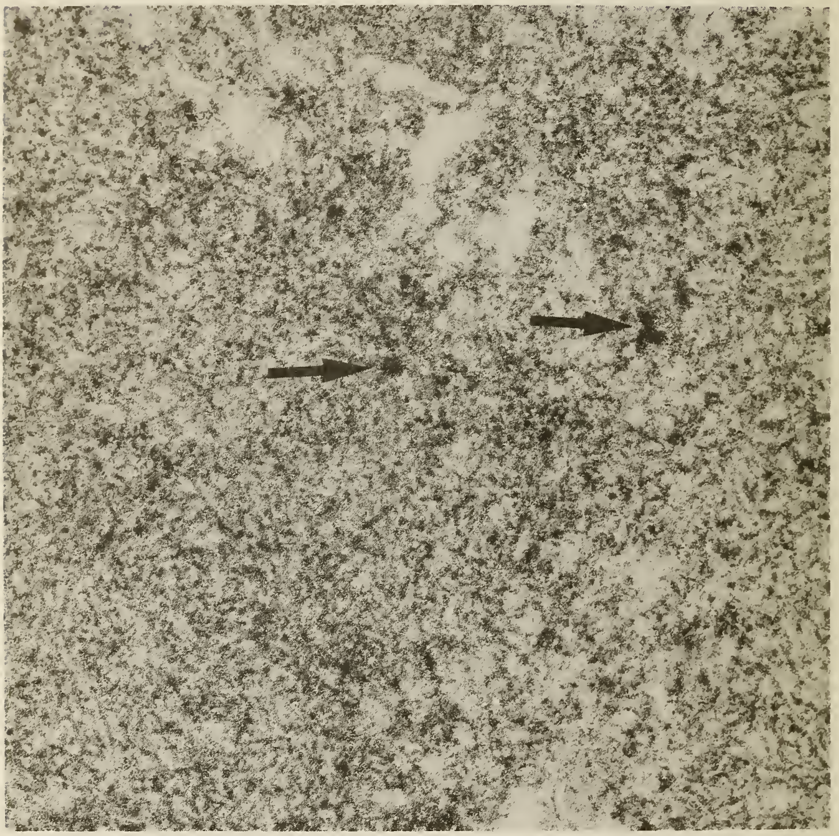


Figure 4. Electron Micrograph of AH Filaments After Deoxycholate Extraction (X62,400)

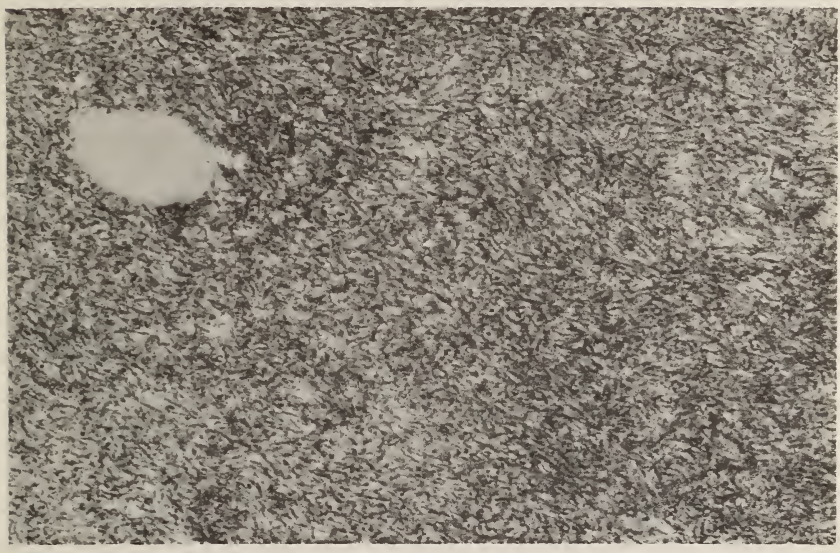
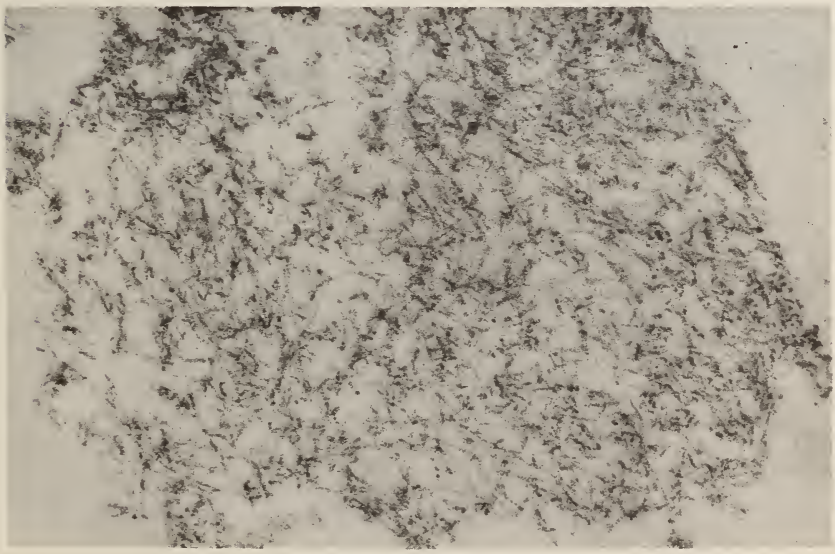
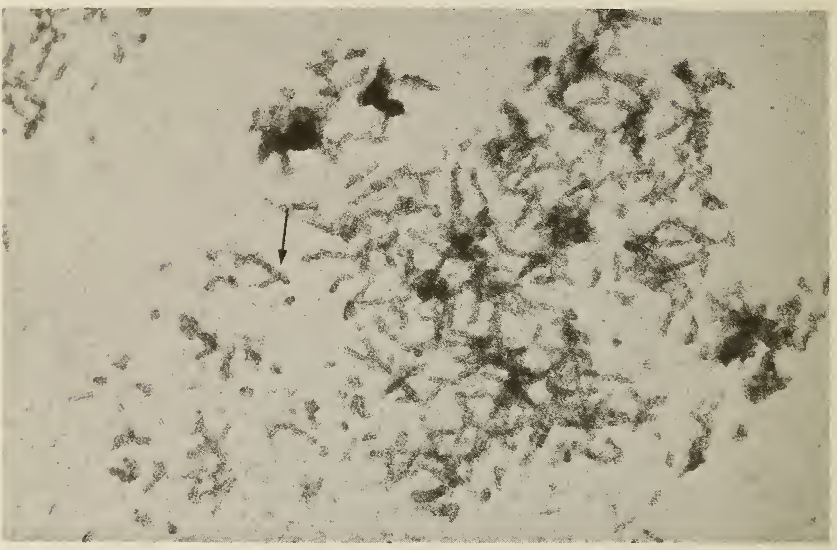


Figure 5. Electron Micrograph of Reconstituted AH Filaments



Note the similarity to AH filaments in figure 4 (X62,400).

Figure 6. Electron Micrograph of Positively Stained Sonicated AH Filaments



Note the branches (arrows) (X97,600).

Figure 7. Higher Magnification of Figure 6 (X157,000)

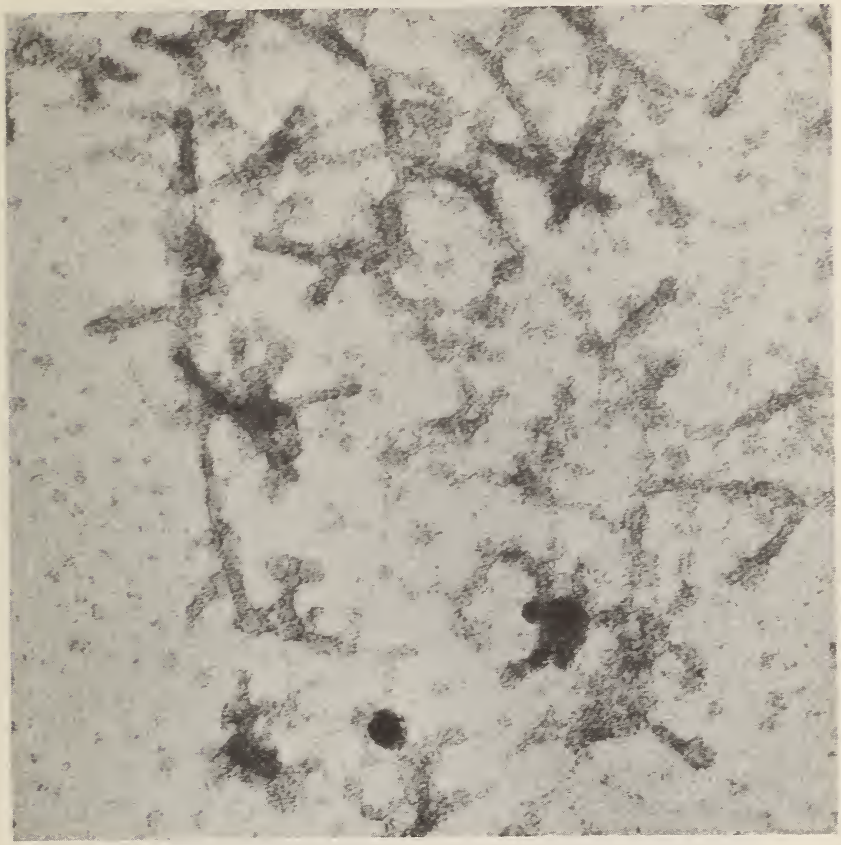


Figure 8. Platinum-Coated AH Filaments (X87,300)

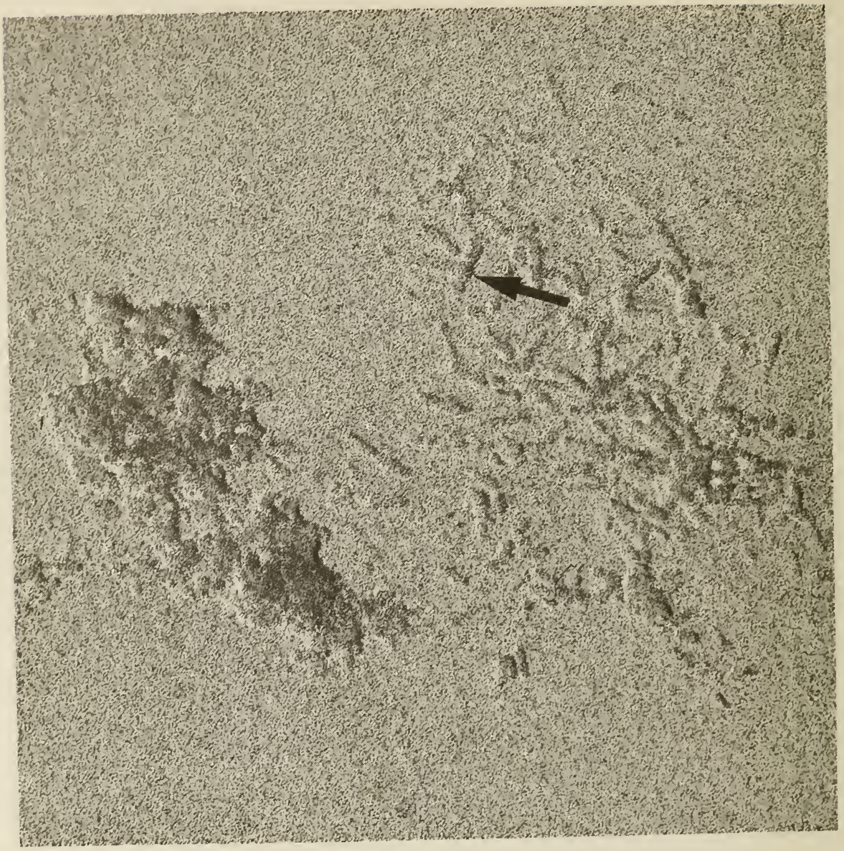
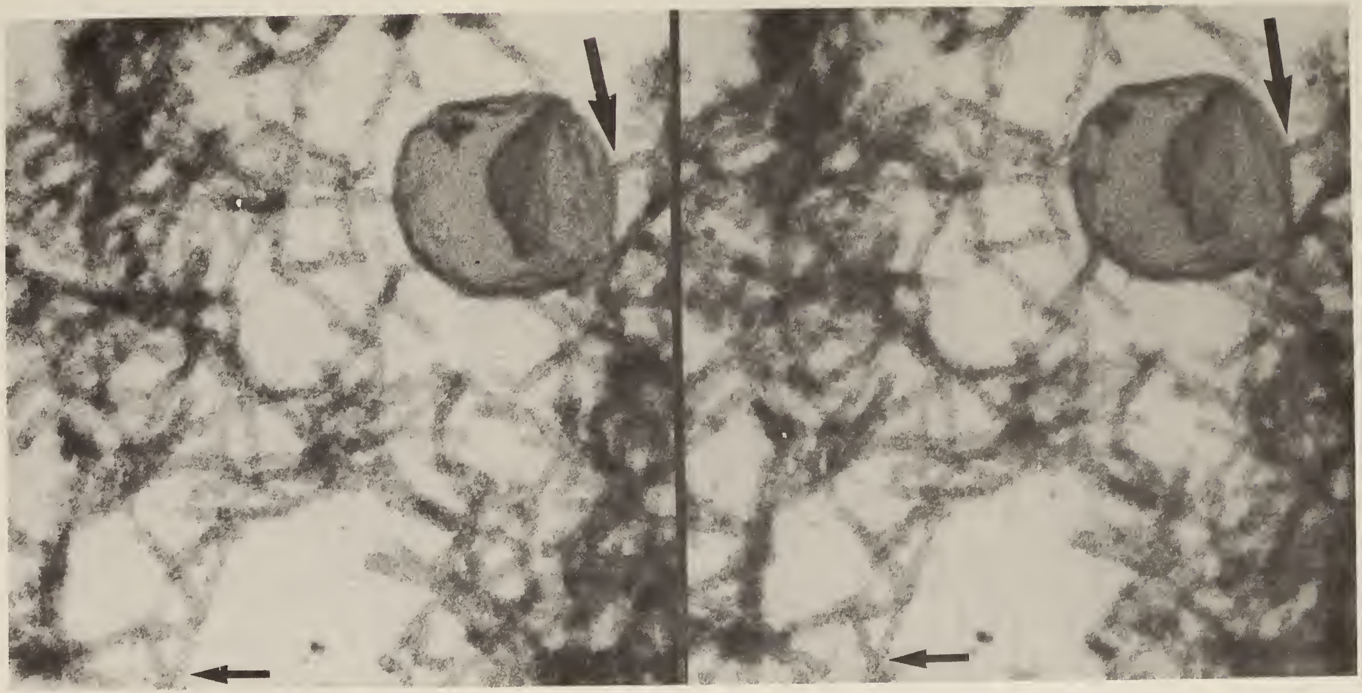


Figure 9. Stereopairs of AH Filaments



Note that the filaments branch (thin arrows) and appear to connect with the membrane of the vesicle (thick arrows) (X142,600).

Table 1. Comparative Molecular Weights of Polypeptide Bands by SDS Gel Electrophoresis (x 10^3)

Substance	Molecular Weights					
	60-66	56-58	50-52	47-48	43	30
Human AH French (in press)		56	50	47	43	30
Mouse AH Franke, Schmid et al. (1979) Franke, Denk et al. (1979)	66,64	57	51	48		
Rat AH Borenfreund, Higgins, and Peterson (1980)	66	57	52	48	43	
Bovine prekeratin Franke et al. (1978)	63,60	58	52,51			

Intracellular Location of Alcoholic Hyalin Filaments and Intermediate Filaments in Human Hepatocytes*

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Introduction

Alcoholic hyalin (AH) filaments are thought to be derived from intermediate filament (IF) proteins of the hepatocyte (Denk, Franke, Eckerstorfer et al. 1979; Denk, Franke, Kerjascki et al. 1979; Franke et al. 1978; Franke, Denk et al. 1979; Franke, Schmid et al. 1979; French and Davies 1975; French, Sim et al. 1977).

The questions then arise: (1) Where in the normal hepatocyte are IFs found? (2) Do the AH filaments occupy the same location as IF? (3) Does the formation of AH interfere with the function of normal IF? To answer these questions, both normal hepatocytes and liver cells containing AH were examined, utilizing liver biopsies from 42 patients suspected of having alcoholic liver disease (ALD).

Method

Liver biopsy specimens from 42 male chronic alcoholics were studied. All biopsies were performed for clinical indications and were obtained after informed consent under protocols approved by the human studies committees of Harbor General Hospital and the Martinez Veterans Administration Medical Center. The morphologic diagnoses were as follows: 5 cases had normal histology; 12 cases showed fatty liver; 2 cases exhibited hepatic fibrosis; 18 cases had alcoholic hepatitis; and 5 cases showed cirrhosis. The diagnosis of alcoholic hepatitis was based on the findings of necrosis, balloon degeneration, neutrophilic infiltration, and alcoholic hyalin. Of the 18 cases with alcoholic hepatitis, 7 cases also had cirrhosis.

The liver tissues were fixed for both light and electron microscopic examination. Hematoxylin-eosin and Masson-trichrome stains were

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NOTE: Figures appear at end of paper.

* This work was supported by the Veterans Administration.

performed to determine the histologic diagnosis. Specimens for electron microscopy were cut into 1 mm cubes and fixed in a solution of 2-percent glutaraldehyde in 0.1 M sodium cacodylate buffer. Afterward, the tissues were postfixed in 1-percent osmium tetroxide in sodium veronal buffer, dehydrated, and then embedded in either araldite or Spurr's low-viscoty resin. Thick sections measuring 1 μ were stained with toluidine blue and used to identify periportal, midlobule, and centrolobular areas and also foci of normal-appearing hepatocytes and hepatocytes containing AH. Selected blocks were trimmed, and then sectioned and stained with uranyl acetate and lead citrate; these were examined by electron microscopy.

Results

AH filaments were found in the same locations as IF in normal hepatocytes. For instance, AH filaments formed a dense aggregate surrounding centrioles (figure 1). In comparison, a rich meshwork of IF was found surrounding centrioles in normal hepatocytes (figure 2). There, IFs were seen oriented to encircle the centrioles and appeared to extend out into the cytosol between organelles. There was a suggestion that IF inserted on the outer membrane of tangentially cut mitochondria (figure 2).

AH filaments were seen to encroach on the nuclear envelopes at many points (figure 3). This relationship to nuclei was sometimes extensive, to the point that AH filaments encircled the nucleus or formed a ring that was focally interrupted. Similarly, a rich meshwork of crisscrossing IFs was noted next to the nuclei of normal hepatocytes. This relationship was best seen when the nuclear envelope was cut tangentially (figure 4). The IFs appeared to connect with nuclear pores (figure 4). When nuclei were cut across the middle, the IF appeared in bundles in the cytosol next to the nucleus, but oriented parallel to the nuclear envelope.

AH filaments sometimes closely approached the plasma membranes (figure 5). When this occurred, no ectoplasm intervened. In contrast, uninvolved hepatocytes showed a thick layer of ectoplasm between organelles in the cytoplasm and the plasma membranes (figure 6). IF ran parallel to the plasma membrane or around the canaliculus in the interface between the ectoplasm and the cytosol (figure 6).

Hepatocytes containing AH were examined to see if there were any associated pathologic changes present in the cells. Changes in the shape of the nuclei (figure 3) were noted. The cell outline was often rounded (figure 7) or bizarre in shape. The organization of organelles in the cytosol was often lost (figure 7). Smooth endoplasmic reticulum appeared increased, and rough endoplasmic reticulum was decreased. The cytosol appeared devoid of the normal IF. At the cell periphery, the ectoplasm appeared to be focally absent, and organelles were closely applied to the plasma membrane (figures 1 and 7) without intervening ectoplasm. Thus, AH formation was associated with loss of nuclear and cellular shape and loss of organization of intracytoplasmic structures—suggesting loss of the normal endoskeleton function.

Discussion

The results indicate that AH filaments are found in the same places within the cell as IF, including juxtanuclear, surrounding centrioles, and in the ectoplasm as well as randomly located in the cytosol. These observations have been reported or depicted by other investigators (Biava 1964; Horvath et al. 1973; Petersen 1977; and Rumpelt 1977); however, the significance of this association was not previously appreciated except by Petersen (1977), who noted that AH filaments connected with IF (microfilaments).

The distribution of normal IF in hepatocytes in man has not been previously reported, except by Sternlieb (1965), who described a network of perinuclear filaments in biopsy specimens of human liver. The distribution of IF in rat hepatocytes has been well characterized by Yokota and Fahimi (1979). They noted "intermediate-sized filaments" of the prekeratin type in four distinct regions of the cytoplasm: (1) in the pericanalicular region; (2) in the ectoplasm below the microvilli of the space of Disse; (3) in associaton with the centriole; and (4) in the perinuclear region abutting on the nuclear envelope. Hence the distribution of IF in rat liver corresponds to that in human liver. IFs have been described in rat liver in studies where a juxtaposition to mitochondria was noted (Fiskum et al. 1980; French and Davies 1975). In the present study, a clear relationship to mitochondria was also noted. The prekeratin nature of hepatocytic IF was established by Franke, Schmid, et al. (1979) using immunofluorescent technique and cell cultures of hepatocytes. These studies showed antigenic cross-reactivity of IF and alcoholic hyalin filaments to an antibody raised against IF proteins of the prekeratin type.

The fact that AH filaments and prekeratin IF share a common antigen permits speculation as to how AH formation may alter the function of affected hepatocytes. The various types of IF function to integrate mechanically the various structures of the cytoplasmic space in a way that is tailored to the differentiated state of the cell (Lazarides 1980). IF (neurofilaments) may be important in plasma membrane-nuclear communications (Metuzals and Mushynski 1974), since IFs extend from the nuclear pores to the plasma membrane. We confirmed the nuclear pore connections in the human hepatocyte. IFs play a role to anchor the nuclei in cultured human fibroblasts (Lehto et al. 1978). IF assembly is also associated with the movement of cytoplasmic organelles (Moellmann et al. 1973). Thus, a variety of IF functions in the hepatocyte might be lost if IF proteins were used up in the construction of AH filaments.

There is evidence that hepatocytic IF is diminished to absent in cells containing AH. Huang and Kimoff (1980), using a monospecific antibody raised against human AH, showed that the antibody bound the ectoplasm and, to a lesser extent, the cytoplasm of normal hepatocytes, as well as AH. In cells containing AH, however, the antibody did not bind the ectoplasm or the cytoplasm. These results are interpreted to mean that IF protein in AH is lost from the ectoplasm and cytoplasm. Petersen (1977) noted fewer IFs in hepatocytes containing AH and suggested that failure of the mitotic spindle to form might result from the loss of IF. The authors' study noted the absence of IF and also focal loss of ectoplasm in hepatocytes that contained AH. This was associated with loss of organization of organelles and the polygonal shape of the cell. Rumpelt (1977) noted that liver cells containing AH were preferentially lysed, suggesting that AH predisposes hepatocytes to undergo necrosis—presumably as a consequence of the loss of endoskeleton.

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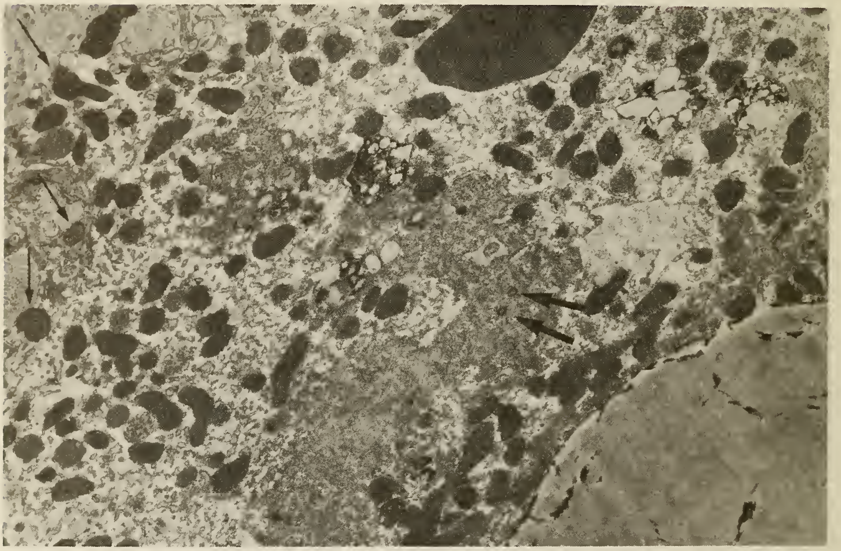
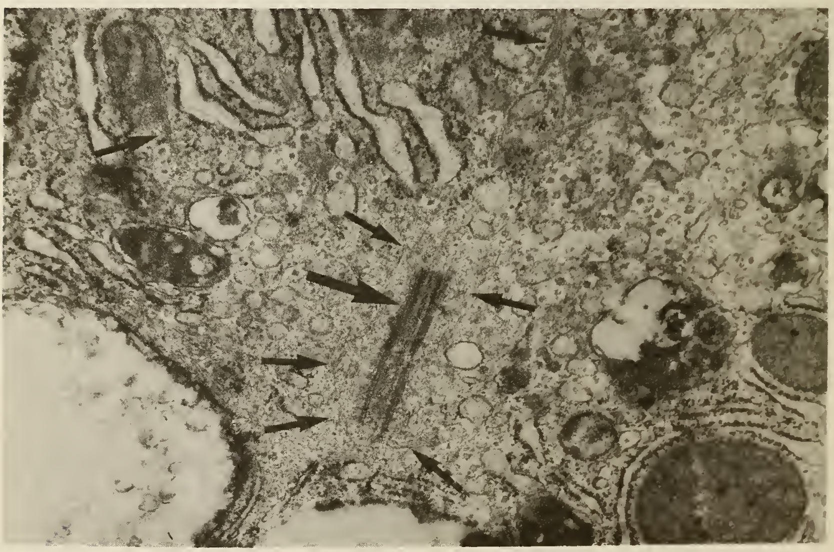
Figure 1. AH Filaments Completely Surround and Enclose Centrioles (Thick Arrows)



Note the associated increase in SER, decrease in RER, and the disarray of organelles in the cytosol. Mitochondria focally encroach on the plasma membrane in the absence of intervening ectoplasm (thin arrows) (X10,900).

Figure 2. Intermediate Filaments (Fine Arrows) Encircle the Centrioles (Thick Arrow) in a Normal Hepatocyte



Note the bundles of IF radiating out through the cytosol between organelles and apparently connecting with mitochondrial membranes cut tangentially (thin arrows) (X29,600).

Figure 3. AH Filaments Surround and Closely Approach a Misshapen Nucleus (Arrows) (X29,600)

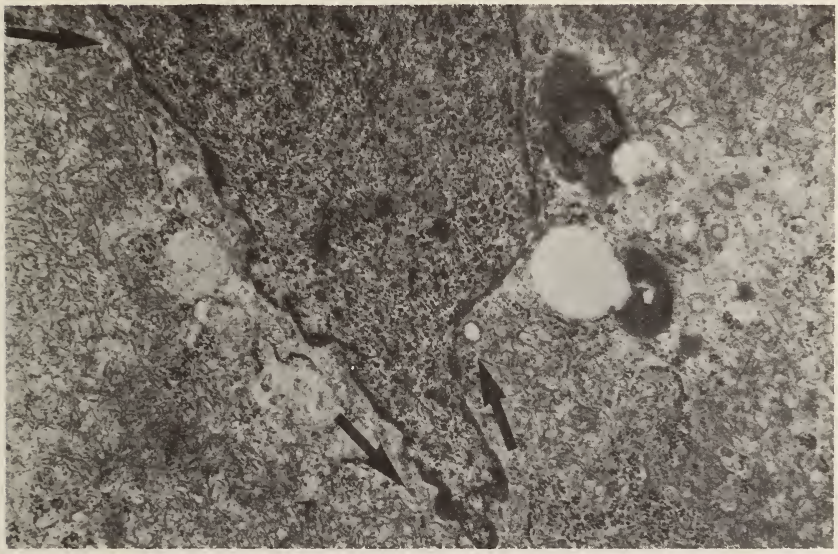
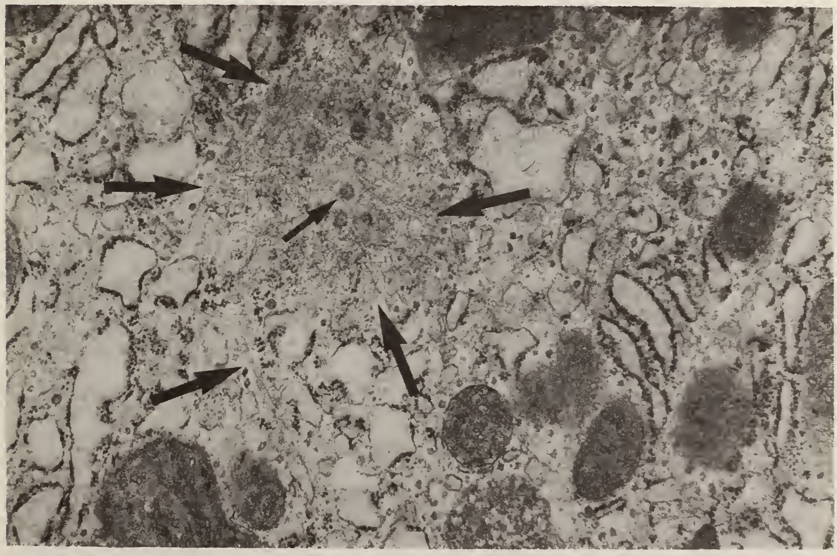
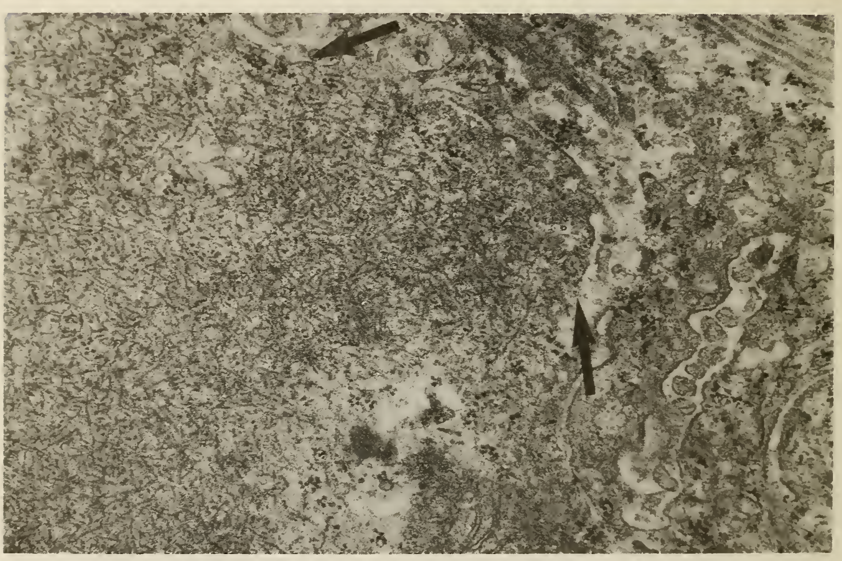


Figure 4. Intermediate Filaments Crisscrossing Next to a Tangentially Cut Nuclear Envelope (Thick Arrows)



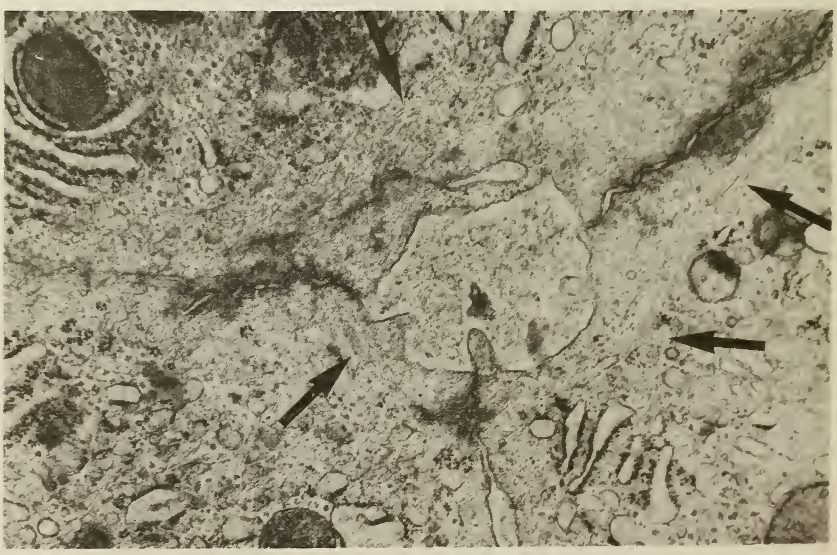
These filaments extend as small bundles between organelles. Some filaments intersect with nuclear pores (thin arrow) (X29,600).

Figure 5. AH Filaments Come Very Close to the Plasma Membrane (Thick Arrows), Without Intervening Ectoplasm



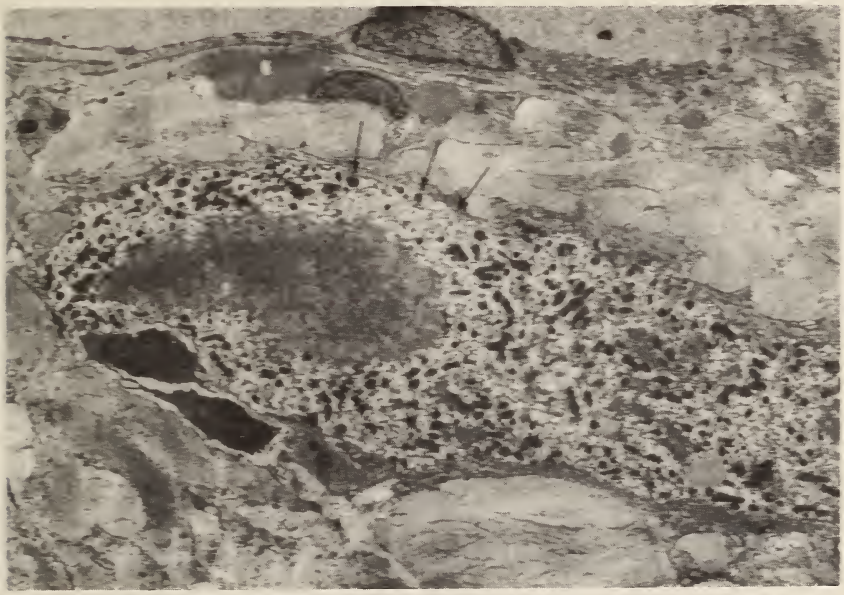
The plasma membrane appears focally disrupted (X42,500).

Figure 6. Intermediate Filaments Encircle the Canaliculus and Run Parallel to the Plasma Membrane in the Interface Between the Ectoplasm and the Cytosol (Thick Arrows)



The canaliculus is dilated and the microvilli are reduced in number (X29,600).

Figure 7. AH Filaments Occupy the Cytosol Without an Apparent Relationship to Any Particular Membrane Structure



Note the washed-out appearance of the cytosol, the random distribution of organelles, the increase in SER, and the loss of ectoplasm (arrows) (X4,125).

SESSION VIII: ANIMAL MODELS

Introduction to Roundtable Discussion of Animal Models

Ting-Kai Li

Abstract

The author points out that in alcoholism research, a number of physiologic, metabolic, and behavioral responses to alcohol can be profitable studies by means of animal models. The paper lists some of these responses, and summarizes the kinds of animal models used in alcoholism research.

Animal Models of Physical Dependence to Ethanol

Carlton K. Erickson

Abstract

This paper lists the criteria of an animal model of physical dependence to ethanol and critiques available models, advising that several species or models are inappropriate or ineffective for producing physical dependence to ethanol in animals. The author discusses a new model, a sustained release model of ethanol exposure that can be used to produce physical dependence in mice in 4 days and rats in 9 days. The author concludes that the unique character of human alcoholism will, by nature, prevent discovery of an ideal animal model of the disease; he suggests that models for individual alcohol-related disorders—such as fetal alcohol syndrome, liver dysfunction, and others—are worthwhile as each of these disorders is so complex it should be studied in isolation.

Animal Models for Alcohol Research

V. Gene Erwin

Abstract

This paper provides an overview of animal models for alcohol research according to alcohol-related behaviors or the actions of alcohol that they most adequately represent. The author discusses voluntary alcohol consumption and ethanol preference; initial central nervous system sensitivity; ethanol metabolism and disposition; alcohol withdrawal and tolerance; and genetically heterogeneous foundation populations.

Animal Models of Liver Injury and Ethanol Dependence in Rats and Baboons

Charles S. Lieber and Leonore M. DeCarli

Abstract

This paper describes a model developed for the administration to rats and baboons of ethanol as part of a nutritionally adequate liquid diet. With this regimen, ethanol intake was much higher than with conventional procedures. All animals gained or maintained their body weight, and liver morphology was normal in the controls. Isocaloric substitution of carbohydrate by ethanol (36 percent of total calories in rats and 50 percent in baboons) resulted in the production of fatty liver in all animals, while the baboons also developed cirrhosis. Inebriation and manifestation of dependence on withdrawal of the diet were observed in baboons and quantitated in the rat. Chemical alterations produced by ethanol at the fatty liver stage were characterized by hyperlipemia, striking triglyceride accumulation in the liver, and enhanced activities of microsomal drug metabolizing enzymes, including the microsomal ethanol oxidizing system (MEOS). In showing that various aspects of liver injury observed in alcoholics can be reproduced in animals by the feeding of pure ethanol with an adequate diet, this study incriminates ethanol itself as a cause for the hepatic complications. This new experimental model is proposed as a tool for the study of the pathogenesis and treatment of alcoholic liver injury and dependence.

ERCP in a Nonhuman Primate: A Model for Teaching and Research

Jerome H. Siegel and Mark A. Korsten

Abstract

This paper discusses endoscopic retrograde cholangiopancreatography (ERCP), a frequently used endoscopic procedure for studying biochemical and morphologic changes in the biliary system and pancreas in humans. Bile and pancreatic juice are obtained in animal models by means of invasive cannulation or fistular production. The authors note, however, that this approach is acceptable in smaller animals but not feasible in larger species such as primates. They report on a study that shows that ERCP can be safely performed in baboons and that juice can be aspirated from each of the ductular systems. This technique permits noninvasive collection of bile and pancreatic secretions and may become a useful research technique in the subhuman primate, they conclude.

SESSION IX: DIAGNOSTIC TESTS

Endoscopic Retrograde Cholangiopancreatography (ERCP) in the Evaluation of Biliary Tract and Pancreatic Disease Associated with Ethanol Abuse

David S. Zimmon

Improved diagnostic efficiency and accuracy in the jaundiced patient is a much sought-after goal. Schenker et al. (1962) presented a prospective analysis of 61 patients to demonstrate that careful history and physical examination alone yielded a diagnostic accuracy of 80 percent for cirrhosis, 77 percent for obstructive jaundice, and 70 percent for hepatitis. If routine laboratory studies were added, diagnostic accuracy in the 20 patients with obstructive jaundice reached 90 percent. Only 7 of 20 patients with obstructive jaundice had a history of alcohol abuse, and only 2 had physical stigmata suggesting cirrhosis such as palmar erythema or spider angiomata. The principle of clinical parsimony was upheld. Since there was little overlap between the patients with intrahepatic cholestasis and those with large bile duct obstruction with respect to these clinical features, the authors felt justified in emphasizing their differential value in determining the cause of jaundice.

Of course, the evaluation of a patient with cholestasis who abuses alcohol must be more complex. These individuals are subject to all the diseases of their abstinent cohorts as well as to numerous nonhepatic diseases related to alcohol such as cholelithiasis and pancreatitis (Dutta et al. 1978; Nicholas et al. 1972). Therefore, apparent hepatocellular disease has less diagnostic value in distinguishing those who have concomitant bile duct obstruction.

Soon after the development of endoscopic retrograde cholangiopancreatography (ERCP), we emphasized the value of this technique as a primary procedure for the diagnosis of biliary tract or pancreatic disease (Zimmon et al. 1975). It provides the options of endoscopic target biopsy, secretory and cytologic studies, culture or chemical analysis, and the therapeutic approaches of endoscopic biliary surgery (papillotomy or sphincterotomy) and placement of stents or drains for the temporary relief of duct obstruction (Zimmon et al. 1980). Fur-

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NOTE: Tables appear at end of paper.

thermore, the hazards are few when compared to percutaneous techniques (Kreek and Balint 1980). Particularly in patients with hepatocellular disease (Goodman et al. 1980), ERCP is the preferred diagnostic modality for investigating suspected biliary tract disease.

Endoscopic retrograde cholangiopancreatography is a combined endoscopic and radiologic procedure that utilizes a specialized fiberoptic endoscope to visualize the upper gastrointestinal tract, identify the papilla of Vater, and under visual control cannulate the pancreatic or biliary duct systems for retrograde injection of radiopaque contrast media. This complex and highly sophisticated procedure has evolved rapidly over the past 10 years. It is a logical extension of advances made through operative and percutaneous transhepatic cholangiography and fiberoptic gastrointestinal endoscopy. Initial attempts to cannulate the papilla of Vater with only radiologic control were made with a curved device fashioned to fit the anatomy of the second duodenum. Almost simultaneously, endoscopists appreciated the fact that the duodenum and the papilla of Vater could be visualized with endoscopes primarily designed for examination of the upper gastrointestinal tract, particularly the stomach. Over a period of 5 years, these two lines of investigation rapidly came together.

In 1970, two Japanese investigators published preliminary experiences with fiberoptic cannulation of the papilla of Vater for the performance of retrograde pancreatography and cholangiography. Initially, it was necessary to perform these procedures under general anesthesia since they were both time-consuming and required control of patient movement and peristalsis in the duodenum. From 1970 to the present, rapid technical advances in the fiberoptic endoscopes used for cannulating the ampulla of Vater and a better understanding of the pharmacology, radiology, and physiology of the duodenum, pancreas, and biliary tree have made ERCP a brief procedure requiring 10 to 15 minutes for the average patient using short-acting intravenous sedation employed for standard upper gastrointestinal fiberoptic endoscopy. This rapid evolution has brought ERCP to the forefront of biliary and pancreatic diagnosis and encouraged its use as a primary diagnostic procedure. Since ERCP is brief and its hazards are few, its use is limited only by the time and effort required to master its technical complexities (table 1).

Indications for ERCP

Cholestasis

In patients with jaundice, visualization of the biliary tree is imperative to exclude mechanical biliary tract obstruction. Particularly in the presence of known liver disease or other potential causes for cholestasis, diagnostic tests available for visualization of the biliary tree are few. Even with bilirubin levels below 3 mg percent, intravenous cholangiography fails to visualize the bile duct in 30 to 40 percent of individuals. Furthermore, visualization of the distal biliary tree by intravenous cholangiography is frequently inadequate. Both false-positive and false-negative diagnostic impressions are common.

ERCP visualized the bile duct in over 80 percent of patients studied and yields dense cholangiograms that provide excellent detail of both extrahepatic and intrahepatic biliary systems. Percutaneous cholangiography has been useful in visualizing the biliary tree in jaundice patients. However, percutaneous cholangiography carries a risk of hemorrhage, sepsis, or failure in a biliary tree without dilated intrahepatic bile ducts. Endoscopic retrograde cholangiography is particularly valuable since the normal biliary tract is easy to cannulate and the risk of retrograde injection of contrast agent into a normal biliary tree is negligible. Thus, complications of ERCP are largely restricted to patients with mechanical duct obstruction. The further advantage of ERCP in providing important information about mucosal or submucosal diseases of the gastrointestinal tract and visualizing the pancreatic duct suggests that it is the procedure of choice for the diagnostic evaluation of a jaundiced patient.

In a seriously ill patient with a rapidly evolving disease process ERCP is particularly valuable. It can be performed in patients who are aged and have sepsis or may be subjected to immediate abdominal surgery. It leaves no delaying barium as an impediment to further diagnostic studies and can be performed in patients prior to the traditional barium enema that is needed to exclude colonic lesions. ERCP can be used when serious underlying disease such as sepsis or hemologic disorders increase the risk of percutaneous transhepatic cholangiography or liver biopsy.

For these reasons, the combination of upper gastrointestinal endoscopy and endoscopic retrograde cholangiopancreatography is invaluable in acutely ill patients suspected of having pancreatic or biliary tract disease. In a consecutive study of 91 patients in which ERCP was used as the primary diagnostic procedure, the origin, nature, or extent of disease was established in 85 percent. A positive clinical diagnosis was established by endoscopy in 13 patients, by pancreatography in 25 patients, and by cholangiography in 39 patients.

Pancreatic Disease

In patients suspected of pancreatic neoplastic disease, retrograde pancreatography has provided a valuable diagnostic tool for delineation of the cause of abdominal pain or jaundice. It is of particular value in older patients, for whom the risk of angiography is great and differentiation between vascular disease and neoplastic infiltration of vessels may be difficult. Perhaps more important is the ability of retrograde pancreatography to differentiate in the majority of cases between pancreatic inflammatory disease and pancreatic neoplastic disease.

Surgical studies comparing operative pancreatography with resected pancreatic specimens have demonstrated that the pancreatic duct system reflects intrapancreatic pathologic processes. As fibrosis of the pancreas increases with the evolution of chronic pancreatic inflammation, the pancreatic duct system dilates. This allows an estimate of residual pancreatic functional mass based on the configuration of the pancreatic duct system. In fact, retrograde pancreatography has replaced the pancreatic secretion test. Studies comparing pancreatic duct morphology determined by pancreatography and pancreatic secretion show a high degree of correlation in patients with chronic pancreatic disease. In acute pancreatitis, the duct system may be relatively intact with diminished pancreatic secretory function that returns toward normal when the acute episode of inflammation has moderated. In patients with acute pancreatitis suspected of biliary tract disease, retrograde pancreatography provides a useful method for visualizing the bile ducts. The pancreatogram in these individuals is usually normal. This is an important clue suggesting the need for a thorough evaluation of the biliary tract and a search for stones in patients with pancreatitis. In patients with acute recurrent or chronic pancreatitis, the retrograde pancreatogram allows the detection or exclusion of obstructing lesions, such as duct strictures or pseudocysts that could be responsible for recurrent attacks of pain and require surgical intervention. Finally, followup pancreatography allows the evaluation of surgical procedures directed at drainage of the pancreatic duct whether via the sphincter of Oddi or by intestinal anastomosis.

Gallbladder Disease

Although oral cholecystography and intravenous cholangiography are useful diagnostic tests for the study of patients with gallbladder disease, the advent of ERCP has added new information. Endoscopy and retrograde cholangiopancreatography allow a precise definition of preoperative anatomy and pathology that may be valuable for the operative management of patients suspected of or having gallbladder disease. Failure to visualize the gallgladder by oral or intravenous routes may result from the presence of a small fibrotic gallbladder in the absence of stones. Such an organ may not require removal. Failure of hepatic contrast excretion or anomalies of the biliary tract may also result in failure to visualize a normal gallbladder. In patients undergoing surgery of the biliary tract, it is valuable to know in advance if there are anomalies of the biliary system, if choledocholithiasis is present or absent, if the pancreatic duct and papilla of Vater are normal, and if occult disease of the gastrointestinal tract's mucosal or submucosal area is present. Endoscopy with endoscopic retrograde cholangiopancreatography makes it possible to approach the ideal in exclusion, or diagnosis, of upper gastrointestinal tract diseases prior to the performance of laparotomy.

Postcholecystectomy Syndromes

The postcholecystectomy syndrome is a diagnostic problem. In the presence of a dilated common bile duct, intravenous cholangiography is rarely adequate to exclude the presence of stones or distal biliary sphincter stenosis. In our hands, retrograde cholangiography and pancreatography have been primarily of value in the exclusion of stones and pancreatic disease in patients who complained of pain after cholecystectomy. Through this technique, unnecessary repetitive biliary tract surgery has been avoided. Occasionally, occult tumors or stones were uncovered. Generally, ERCP has served to exclude objective disease in a complaining patient.

Abdominal Pain

In the patient with chronic abdominal pain, endoscopic pancreatography is extremely valuable and unique in being able to demonstrate pancreatic neoplasm and pancreatic inflammatory disease without the necessity of abdominal exploration. In addition, high-quality cholangiography and visualization of the gallbladder occasionally uncover cholelithiasis, or choledocholithiasis, that has been overlooked by oral cholecystography or intravenous cholangiography.

In patients acutely ill with abdominal pain, ERCP is invaluable in allowing a positive diagnosis of upper gastrointestinal tract pathologic processes, or after excluding upper gastrointestinal disease, leaving the field free for a further diagnostic evaluation by intravenous pyelography, angiography, or colonoscopy, and barium enema.

Therapeutic Endoscopy

Therapeutic endoscopy is a rapidly evolving field at the moment directed at the management of choledocholithiasis and distal biliary tract stenosis. The ability to instrument the biliary tree allows the injection of antibiotics and mechanical flushing of the biliary tree to relieve sepsis and remove small biliary tract stones. Instruments have been developed for the extraction of stones and for the performance of endoscopic papillotomy and sphincterotomy to remove stones and relieve stenosis. Early reports suggest that transduodenal endoscopic surgery is the standard method for the management of choledocholithiasis and distal biliary tract stenosis. Endoscopic stents and drains allow the safe peroral decompression of a biliary tree obstructed by stones or stenosis of inflammatory or neoplastic origin.

Complications

The complications of ERCP are largely related to the underlying pathology. During the initial development of this procedure, drugs were required in large doses to achieve adequate sedation and to produce sufficiently long duodenal aperistalsis for cannulation to be performed. With increasing skill, the duration of the procedure has become brief, reducing the quantity of sedative required. Anticholinergics have largely been replaced by glucagon. With the use of proper endoscopic techniques retrograde cholangiography and pancreatography in the presence of normal duct systems are essentially without risk. Since ERCP is an endoscopic procedure, the hazard of general endoscopy—including aspiration, perforation, and the like—applies to it as to any endoscopic procedure.

We have recently reviewed our experience with 1,089 attempts at retrograde cannulation of the papilla of Vater. In that group of patients, there were two serious septic complications leading to death. In 1 patient, gram-negative sepsis, in the presence of carcinoma of the pancreas that precluded drainage of the biliary tract, did not respond to antibiotic therapy. In a patient with choledocholithiasis and both pancreatic and common duct obstruction, delayed surgical intervention in a poor-risk patient led to a late death from intra-abdominal sepsis. It is important to recognize the need for early surgical intervention as treatment with antibiotics both locally and systemically may fail to control sepsis. In 235 patients with pancreatic disease, 8 patients (3.5 percent) experienced brief self-limited exacerbation of pancreatitis characterized by fever, hyperamylasemia, or abdominal pain after retrograde pancreatography. Although all these patients were prepared for drainage of pancreatic lesions if present, on no occasion was this necessary. All of these patients responded promptly to antibiotic therapy and gastric suction. Examples of serious pancreatitis following retrograde pancreatography have been reported in the medical literature. All of our patients are prepared for surgical intervention and followed closely by the surgical staff.

Diagnostic Yield

The diagnostic success rate for ERCP has remained at approximately 75 percent over a series of more than 1,000 patients. As listed in table 2, the diagnostic yield of ERCP is the sum of endoscopic findings, cannulation of both pancreatic and biliary ducts, or cannulation of a single diagnostic duct system.

Conclusion

Endoscopic retrograde cholangiopancreatography is a demanding technique requiring a considerable expenditure of time and effort for its mastery. This effort is repaid by a rapid, accurate diagnostic yield with less risk than any procedure of similar diagnostic spectrum. The rapid development of therapeutic endoscopy for the treatment of choledocholithiasis and biliary-tract stenosis adds an important therapeutic modality to this useful diagnostic technique.

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Table 1. Diagnostic and Therapeutic Spectrum of Endoscopy with ERCP

I. Fiberoptic Endoscopy (Esophagus to Jejunum)
  1. Mucosal Diseases
    A. Inflammation (esophagitis, gastritis, duodenitis)
    B. Neoplasia
  2. Submucosal Diseases
    A. Esophagogastric varices
    B. Neoplasia
    C. Inflammation (pancreatitis)
  3. Papilla of Vater
    A. Location
    B. Lumen
    C. Pathology (inflammation, neoplasia)
  4. Diagnostic Aids
    A. Mucosal target biopsy
    B. Cytologic, brush, or fluid samples
    C. Bile analysis or culture
    D. Pancreatic secretion
II. Endoscopic Retrograde Pancreatogram
  1. Pancreatic inflammatory disease
  2. Pancreatic neoplasia
  3. Preoperative and postoperative pancreatic duct anatomy (plan surgery, avoid duodenotomy and pancreatography; confirm success of drainage, progress of disease)
III. Endoscopic Retrograde Cholangiogram
  1. Extrahepatic biliary tree—lithiasis, neoplasia, stricture, variant anatomy
  2. Cystic Duct and gallbladder—lithiasis, neoplasia
  3. Intrahepatic biliary tree—infiltration, cirrhosis, abscess, neoplasia
IV. Therapeutic Endoscopy
  1. Lithiasis
  2. Distal biliary stenosis
  3. Cholecystitis
  4. Cholangitis
  5. Neoplastic stenosis

Table 2. Diagnostic Value of ERCP in 1,089 Attempts

	Percent
Diagnostic success overall	75.2
By endoscopic findings	4.4
By cannulation	
Both ducts	45.4
Single diagnostic duct	25.4
Diagnostic failure overall	24.8
Cannulated insignificant duct	10.5
Cannulation failures	
Anatomic	6.8
Pharmacologic	7.5

Medical Diagnostic Tests for Alcoholism

Frank A. Seixas

Abstract

This paper discusses the search for specific chemical tests to establish the diagnosis of alcoholism stimulated by publication of the National Commission on Alcoholism (NCA) Criteria for the Diagnosis of Alcoholism.

In discussing various tests the author observes that: tests that measure the acute drinking episode are only useful in diagnosing alcoholism if they establish the presence of tolerance; a test that is claimed to demonstrate prolonged drinking is undergoing extensive evaluation; a test has been discovered that may be a result of alcoholism or perhaps demonstrate a susceptible individual; many metabolic alterations accompany alcoholism, but have not been standardized as tests; some tests are good, some poor, for uncovering relapses; and specific tests have been developed for individual disease consequences of alcoholism.

The author reports that one of the most promising directions is evaluation of the pattern of abnormalities in usually performed tests that lead to a diagnosis of alcoholism, explored by two recent investigators with success. He concludes that establishing a biochemical test for alcoholism will not only make the test of treatment referral easier, but will also reinforce the concept of alcoholism as a disease.

Ethanol and Acetaldehyde: Blood vs. Breath Determinations

Pekka Jauhonen, Enrique Baraona, and Charles S. Lieber

Abstract

This paper reports on a research study designed to evaluate the use of breath acetaldehyde analysis to estimate blood acetaldehyde concentrations in view of their previous finding of acetaldehyde production from ethanol in the respiratory tract. After ethanol administration, 11 baboons were catherized via the femoral vein into right atrium and hepatic vein and breath samples collected both excluding and including the upper respiratory tract. A great overestimation of blood acetaldehyde was found when total breath acetaldehyde was used for calculation of blood acetaldehyde concentrations. If, however, the contribution of upper respiratory tract was avoided by sampling with an endotracheal tube, excellent agreement with blood acetaldehyde levels was found. Correlation between end-expiratory air acetaldehyde and acetaldehyde concentrations in peripheral veins was also studied in 13 human volunteers, 6 chronic smokers and 7 nonsmokers. Overestimation of blood acetaldehyde concentrations resulted when acetaldehyde in end-expiratory air was used for the calculations. This overestimation was greatly exaggerated in smokers. Pyrazole mouthwash prior to breath sampling diminished the contribution of the upper respiratory tract in both groups, but its effect was only partial, especially in the smokers. The authors concluded that breath acetaldehyde analysis should not be used as a substitute for direct blood acetaldehyde analysis.

Optimal Use of Plasma Alpha Amino-n-Butyric Acid and Gamma Glutamyl Transpeptidase for the Detection of Alcoholism

Spencer Shaw, Barry Stimmel, David Korts,
Theresa M. Worner, and Charles S. Lieber

Abstract

The authors report on a study designed to evaluate the optimal use of plasma AANB concentration and GGTP activity for the detection of alcoholism in two populations characterized by a high incidence of alcoholism as well as liver disease: 165 methadone maintenance patients and 90 alcoholics treated for medical complications of alcoholism. The study demonstrated that GGTP and AANB measures for detecting alcoholism in patients with liver disease are used optimally when follow-up values are compared to individual baseline values determined during abstinence. The combined use of the two measures detected a majority of alcoholics (80 percent) with only 12-percent false positives.

Diagnostic Tests in Alcoholism

Theresa M. Worner and Charles S. Lieber

Abstract

This paper discusses prognostic factors associated with progression of alcoholic liver injury, and notes that glutamate dehydrogenase (GDH) has been identified as a reliable marker of liver cell necrosis. A preliminary study indicates that a chronic evaluation of GDH portends a poor clinical outcome. The authors conclude that a plasma GDH elevation is useful in predicting liver cell necrosis, although sampling must be done early since levels rapidly return toward normal; liver function tests (SGOT and SGPT) are not useful in predicting long-term outcome. The authors also discuss perivenular fibrosis (PVF) as a prognostic factor and report on a study of 20 male alcoholics designed to define the histologic progression of this lesion. Presence of perivenular fibrosis appears to portend a poor clinical outcome in male alcoholics; histologic features were prognostically helpful, but routine lab tests performed at the time of the initial biopsy were not. The authors advise that patients with PVF at the fatty liver stage should be monitored carefully and cautioned about the high risk of progression of liver disease if abstinence is not maintained.

Significance and Clinical Detection of Hepatic Fibrosis in Chronic Alcoholics

Yasushi Hasumura, Masanobu Nishimura,
Yutaka Kaku, and Jugoro Takeuchi

Abstract

This paper reports on the national surveillance study of 94 hospitals in Japan that disclosed that the incidence of alcoholic liver disease has been increasing recently, concomitant with an increase of alcoholics with hepatic fibrosis. Alcoholic fibrosis is now considered to be the precursor of alcoholic cirrhosis in Japan. It has been found that the diagnosis of alcoholic fibrosis and its differentiation from fatty liver is usually possible by a combination of the scoring analysis of both clinical and laboratory findings, and of the measurement of serum glycoprotein changes after an oral administration of a small dose of alcohol.

SESSION X: TREATMENT OF ALCOHOLIC LIVER DISEASE

Propylthiouracil (PTU) in the Treatment of Alcoholic Liver Disease

Yedy Israel and Hector Orrego

Chronic alcohol consumption leads to the development of metabolic tolerance, a condition in which the oxygen requirements of the liver are increased (Israel, Videla, and Bernstein 1975; Khanna and Israel 1980). This state—which we have termed "hypermetabolic state"—is dependent on thyroid hormone function and can be abolished or can be markedly reduced by antithyroid drugs or by thyroidectomy (Israel, Videla, and Bernstein 1975; Israel, Kalant, Orrego, Khanna, Videla, and Phillips 1975). This condition of increased oxygen utilization can be observed even in the absence of alcohol (Israel, Videla, and Bernstein 1975). We have postulated (Israel, Kalant, Orrego, Khanna, Videla, and Phillips 1975) that an increase in oxygen consumption in cells accentuates the relative state of hypoxia in areas around the terminal hepatic vein ("central vein"; zone 3 of the liver acinus), a situation that would lead to cell damage when there is a concomitant reduction in oxygen availability. This hypothesis was tested in the chronically alcohol-fed rat subjected to three different conditions that reduce the availability of oxygen to the liver: hepatic artery ligation (Kalant et al. 1975), experimental anemia (Israel et al. 1977), and exposure of the animals to atmospheres with low oxygen tensions (Israel, Kalant, Orrego, Khanna, Videla, and Phillips 1975). These three conditions resulted in hepatocellular necrosis in zone 3 of acinus only in ethanol-fed animals. Propylthiouracil, an antithyroid drug, was tested in animals submitted to hypoxia (Israel, Kalant, Orrego, Khanna, Videla, and Phillips 1975). This drug was found to suppress the hypoxic liver damage produced in the ethanol-fed animals.

Several lines of evidence suggest that periacinar hypoxia might also be the mechanism by which the alcoholic develops liver damage in zone 3 of the acinus. Alcoholics show marked metabolic tolerance to alcohol, up to 100 percent (Bode et al. 1979; Iturriaga et al. 1980; Kater et al. 1969; Mendelson et al. 1965; Ugarte and Valenzuela 1971), and such an increase in the rate of ethanol metabolism, associated to low oxygen tensions in the hepatic vein, appears to be related to the production of hepatocellular necrosis (Iturriaga et al. 1980; Ugarte et

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NOTE: Tables appear at end of paper.

al. 1972). Recently abstinent alcoholics (Iturriaga et al. 1980; Kessler et al. 1954) and nonhuman primates (Shaw et al. 1977) show reduced oxygen tensions in the hepatic vein. Such a relative liver hypoxic state can be accentuated in several physiological and pathological conditions. Alcoholics are exposed, at a higher frequency than normal individuals, to a variety of situations that lead to a reduction in liver oxygenation, including respiratory and pulmonary dysfunction (Emirgil et al. 1974), pneumonia (Smith and Palmer 1976), a reduction in liver blood flow upon alcohol withdrawal (Iturriaga et al. 1980; Kessler et al. 1954), and anemia due to nutritional and metabolic effects (Eichner 1973; Hines and Cohen 1974). Anemia is more likely to occur in females during the reproductive years, where anemia per se is more prevalent (Wintrobe 1974). In a general population of alcoholics, anemia was found to be present in 4 percent of males and 13 percent of females (Wilkinson 1980). It is of interest that recent studies have shown that although the prevalence of cirrhosis is higher in young alcoholic women than in men (Wilkinson 1980; Schmidt and Popham 1980), this relationship is reversed in women over 50 years old (Schmidt and Popham 1980) whose menstrual cycles have ceased. In a sample of patients with alcoholic hepatitis presenting to a general hospital in the United States, 43 percent of the patients had hematocrit values below 30 percent (Lischner et al. 1971). The probability of a combination of several factors reducing oxygen delivery to the liver in addition to anemia is a real one in alcoholics.

Studies presented above support the idea that the (human) alcoholic hepatocellular necrosis in zone 3 might also be of hypoxic nature. If this is the case, propylthiouracil would also be beneficial in the treatment of alcoholic hepatitis in humans. The use of propylthiouracil in hospitalized patients, however, has some constraints. Studies have shown that metabolic tolerance in alcoholics disappears 2 to 3 weeks after alcoholic withdrawal (Bode et al. 1979; Kater et al. 1969; Mendelson et al. 1965; Ugarte and Valenzuela 1971). Thus, a hypoxic state in the liver caused by increased oxygen utilization is also expected to have a roughly similar time course. Therefore, antithyroid drugs are expected to be effective in hospitalized patients who have stopped consuming alcohol only if the drugs are given shortly after alcohol withdrawal. The major benefit of these drugs is expected to be in ambulatory patients who do not abstain, a frequent occurrence among alcoholics with liver disease (Orrego, Blendis, Blake, Kapur, and Israel 1979).

A clinical trial on the effectiveness of propylthiouracil (PTU) in hospitalized alcoholics with liver disease was conducted in 133 patients with an average abstinence of 1 to 2 days (Orrego, Kalant, Israel, Blake, Medline, Rankin, Armstrong, and Kapur 1979). Patients were randomly assigned to a double blind placebo controlled trial lasting a maximum of 45 days. In patients in whom coagulation permitted, a liver biopsy was performed and diagnosis by light microscopy was available. The study was not limited to those with biopsies because of the bias that this selection criterion would have entailed. Therefore, the criteria for assessing the effectiveness of PTU were functional rather than morphological.

One of the problems in assessing the action of any treatment for liver disease refers to the selection of criteria that would permit quantitative comparisons of response to the two treatments that would not be disproportionately influenced by any single clinical or laboratory measurement. Therefore, a composite clinical and laboratory index (CCLI) was developed (Orrego, Kalant, Israel, Blake, Medline, Rankin, Armstrong, and Kapur 1979). The scoring system is based on the concept that the severity of the disease is in proportion to the number of abnormal clinical and laboratory findings. A validity of the CCLI index has been presented previously (Orrego, Kalant, Israel, Blake, Medline, Rankin, Armstrong, and Kapur 1979). Table 1 shows the scale for CCLI scoring. The maximum severity score is 26.

To assess the effect of treatment, a "normalization rate" was devised (Orrego, Kalant, Israel, Blake, Medline, Rankin, Armstrong, and Kapur 1979). Two patients hypothetically might attain the same final degree of improvement but take different times to do so. Normalization rate represents the difference between the highest and lowest CCLI scores in each patient, divided by the number of days it takes to reach the lowest score, and multiplied by 100 for convenience. The higher the normalization rate, the faster is the recovery.

Propylthiouracil treatment markedly increased the rate and extent of normalization of the CCLI in patients with alcoholic hepatitis and also among those severely ill on whom a biopsy could not be performed because of abnormal prothrombin time (table 2). As expected, PTU was not effective in patients with inactive cirrhosis who did not have necrosis and inflammation. Our data did not allow a clear definition on the effect of PTU in patients with fatty liver (table 2). The marked beneficial effect of PTU in severely ill patients with abnormal prothrombin suggested that the effect of PTU might depend on the severity of the disease. This hypothesis was confirmed when patients were divided into two groups according to the initial CCLI, corresponding to the lower and upper halves of the potential scoring range (1 through 13 and 14 through 26). PTU was effective only in the more severely ill group. Further, it was found that PTU at the dose given (300 mg/day) elevated TSH levels only in the severely ill group, thus suggesting that a hypothyroid action of PTU is indeed necessary for its beneficial activity on liver disease (Israel, Walfish, Orrego, Blake, and Kalant 1979).

Table 3 shows the effect of PTU on the normalization rate of each parameter included in the CCLI that was amenable to quantification (Orrego, Kalant, Israel, Blake, Medline, Rankin, Armstrong, and Kapur 1979). As can be observed, PTU not only improved the recovery as measured by the combined index (table 2), but it also enhanced the normalization of most of the individual tests comprising the CCLI (table 3).

The only complication specific of PTU therapy was a leukopenia in one patient after 21 days of treatment, which resolved within 2 weeks of discontinuing the drug. Skin rashes were found with equal frequency in the PTU and placebo groups.

PTU did not significantly affect the mortality rate in this study. Four patients died in the PTU group; the mortality in the placebo group was 6 patients. This rate of mortality would necessitate a study with more than 600 patients in order to derive a statistically significant difference not subjected to a type II error (Orrego et al. in press).

Although this acute study of the effect of PTU was most promising, it is not possible to infer from it the effect of PTU on the long-term prognosis of alcoholic liver disease. A study in which PTU is administered to ambulatory patients is currently being undertaken. Such a study will obviate many of the constraints of a study of PTU therapy in hospitalized patients. In the latter, PTU should be administered shortly after alcohol withdrawal because it is not expected to act in patients whose metabolic tolerance has subsided. Further, PTU has a lag time of its own, required to substantially reduce the circulatory levels of thyroid hormones. Therefore, PTU is not expected to act in comatose patients or those with hepatorenal syndrome for whom death is imminent. In further studies, primarily of ambulatory patients, the dose and length of administration of PTU should be titrated to a given level of increase in TSH, as a reflection of hypothyroid state.

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Table 1. Scale for Composite Clinical and Laboratory Index Scoring

Clinical			Laboratory		
Sign or Symptom		Score	Laboratory Test		Score
Hepatomegaly		1	Serum glutamic oxalacetic transaminase	(>20 U)	1
Splenomegaly		1			
	Grade		Serum gamma glutamyl peptidyl transferase		
Ascites	1 +	1		(>100 U)	1
	2 +	2		(>200 U)	2
	3 +	3			
	Grade		Akaline phosphatase	(>80 U)	1
Encephalopathy	I	1	Serum albumin	<2.59 percent	1
	II	2			
	III	3			
Bleeding tendency (clinical)		1	Prothrombin time (sec above control)		
Spider nevi		1		<3	1
Palmar erythema		1		3.1–5	2
Collateral circulation		1		>5	3
Peripheral edema		1	Bilirubin	1.2–2 mg percent	1
Anorexia		1		2.1–5 mg percent	2
Weakness		1		>5 mg percent	3

(From Orrego, H.; Kalant, H.; Israel, Y.; Blake, J.; Medline, A.; Rankin, J.G.; Armstrong, A.; and Kapur, B. Effect of short-term therapy with propylthiouracil in patients with alcoholic liver disease. *Gasteroenterology*, 76(1):105-115, 1979. Copyright 1979 by Elsevier North-Holland Inc. [NY].)

Table 2. Relation Between Histological Diagnosis and Effect of Treatment With Propylthiouracil

Histological Diagnosis	Treatment	Change in Score (initial-lowest)	Days to Plateau	Normalization Rate[a]
Fatty liver	PTU (n = 13)	5.4 ± 0.5[b]	14.2 ± 2.6	48.9 ± 9.4
	Placebo (n = 6)	2.7 ± 0.6	10.5 ± 1.6	23.8 ± 6.9
	p	<0.025	N.S.	N.S.
Alcoholic hepatitis	PTU (n = 19)	5.5 ± 0.3	13.6 ± 1.2	43.6 ± 4.6
	Placebo (n = 19)	3.5 ± 0.2	18.8 ± 1.7	19.8 ± 3.3
	p	<0.05	<0.01	<0.001
Cirrhosis without hepatitis	PTU (n = 10)	5.2 ± 0.6	14.0 ± 2.5	42.1 ± 5.7
	Placebo (n = 11)	4.9 ± 0.6	15.5 ± 1.9	30.5 ± 4.1
	p	N.S.	N.S.	N.S.
Abnormal prothrombin time[c]	PTU (n = 15)	4.1 ± 0.9	15.1 ± 1.4	32.9 ± 6.9
	Placebo (n = 10)	1.3 ± 1.0	18.2 ± 2.9	2.6 ± 3.7
	p	<0.05	N.S.	<0.005

[a] See text.

[b] Mean ± SE.

[c] More than 3 sec above controls.

(From Orrego, H.; Kalant, H.; Israel, Y.; Blake, J.; Medline, A.; Rankin, J.G.; Armstrong, A.; and Kapur, B. Effect of short-term therapy with propylthiouracil in patients with alcoholic liver disease. *Gastroenterology*, 76(1):105-115, 1979. Copyright 1979 by Elsevier North-Holland, Inc. [NY].)

Table 3. Normalization Rates of Different Clinical and Laboratory Abnormalities in Patients With Alcoholic Hepatitis and With Abnormal Prothrombin

	Alcoholic Hepatitis		Abnormal Prothrombin	
	Placebo	PTU	Placebo	PTU
Hepatomegaly (cm) $\left(\frac{\Delta \text{cm}}{\text{days}} \times 100\right)$	19	40	16	30
Hepatomegaly (cm)	4.0	15	3.0	17
Ascites (0 – 3 +) $\left(\frac{\Delta +}{\text{days}} \times 100\right)$	5.0	12	–5.0	15
Encephalopathy (0 – 4 +)	--	--	0	10
Peripheral Edema (0 – 3 +)	1	15	3	18
Alkaline Phosphate (u) $\left(\frac{\Delta \text{u}}{\text{days}} \times 100\right)$	66	75	502	545
SGOT (u)	52	530	93	100
SGGPT (u)	558	1505	42	1693
Albumin (gm/dl)	–3.0	3.0	–1.0	2.0
Prothrombin (secs)	--	--	–9.0	35
Bilirubin (mg percent)	5.0	15	–6.0	23

Adrenocorticosteroid Therapy in Alcoholic Hepatitis: The Need for and the Difficulties in Combining Data*

Harold O. Conn

Controlled clinical trials do not always answer the questions they were designed to answer. Occasionally, the results of different investigations reach discordant conclusions. Often studies show trends, but the numbers of patients studied are too small to achieve statistical significance. One is tempted in such circumstances to combine the results of the individual studies in hope of finding an answer not available from the studies themselves.

The use of adrenocorticosteroids (ACS) in alcoholic hepatitis is one such situation. Ten controlled clinical trials in which 345 patients were randomized have been reported (table 1). In two of the studies (Helman et al. 1971; Lesesne et al. 1978), ACS therapy was associated with a highly significant reduction in mortality, and in two others there was a statistically insignificant trend in the same direction. In one, the opposite trend was observed. In the other five, the survival pattern was similar in the two groups.

Such results can cause consternation. Assuming that steroid therapy has no effect on survival in alcoholic hepatitis, one might expect that 1 of 20 studies would by chance show a statistically significant difference at the 5 percent level. Is 2 of 10 statistically different from 1 of 20? Since a difference might by chance have occurred equally well in favor of placebo therapy, these results would appear not to have occurred by chance ($p < 0.05$). Somehow, this type of analysis is not too reassuring; one wonders why the other 8 went wrong. Furthermore, controlled clinical trials, which involve many investigators, are complicated, difficult to sustain, and extremely expensive. It may not be possible to continue all investigations until they achieve a satisfactory endpoint, or to mount large new studies designed to answer unanswered questions.

The alcoholic hepatitis-steroid quandary seems to be an ideal situation for combining. When the deaths among the 181 patients selected

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NOTE: Figures and tables appear at end of paper.

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to receive placebo therapy are totaled, the percentage who died (48 percent) is similar to that observed among the 164 who received steroid therapy (43 percent) ($\chi^2 = p > 0.10$). This summation method of combining appears to be a satisfactory solution to a difficult problem. Purists who oppose such combinations can restrict their analyses to the results of the 10 investigations and can attempt to apply the diverse results to their own patients. Others might consider the individual studies and, in addition, note the negative findings when all the data are summed. Summary readers might conclude that there is no place for ACS therapy in alcoholic hepatitis.

Four questions should be asked about combining data:

1. *Can results be combined*? Of course they can! They are combined in table 1. This, however, doesn't make it correct, or provide simple solutions to complex questions.

2. *Should the results be combined*? Some statisticians believe they should be combined when the results are homogeneous, but not when they are heterogeneous (Elashoff 1978).

I accept the first portion of this concept, but reject the second. If the therapeutic results are sufficiently homogeneous, there is little need to combine the data. The conclusions are obvious. Sometimes, however, combination of consonant but inconclusive data may be helpful.

When the therapeutic results of various studies are discordant, combining data may be necessary in order to come to a conclusion, although it may be difficult to reach a consensus.

In combining data, it is far more important that the clinical material be uniform than that the results be uniform. It makes little sense to introduce heterogeneous data and expect to reach homogeneous conclusions. The combining of dissimilar data may be considered a corollary of the computer dictum, "Garbage in, garbage out."

Examination of the 10 controlled trials indicates that they are extremely heterogeneous. Some of the patients in the different studies differed in virtually all demographic characteristics. Although most were in the same age range, the youngest group averaged 41 years (Lesesne et al. 1978) and the oldest, 59 (Schlicting et al. 1976) (table 2). Prognosis must certainly vary in such groups.

Sex, as usual, reared its head. In one series, all of the patients were male (Blitzer et al. 1977); in another, more than two-thirds were women (Helman et al. 1971) (table 2). Sex is a most important risk factor, since it has been shown previously that women with alcoholic liver disease are much more responsive to ACS therapy than are men (Copenhagen Study Group for Liver Diseases 1974).

Furthermore, some of the studies differed in design and execution. One of the studies required liver biopsy (Helman et al. 1971), which by virtue of its requirement for near normal coagulation imposes a series of unintentional criteria of inclusion (table 2). In effect, this criterion probably excluded from the investigation those patients with the most severe alcoholic hepatitis. One of the studies was randomized, but did not employ placebos (Campra et al. 1973). In several others, patient allocation was not truly randomized but was based on the oddness or evenness of the patients' dates of birth (Schlicting et al. 1976; Schumaker et al. 1978).

Different adrenocorticosteroids were used by different investigators (table 2). The main difference was between the use of prednisone and prednisolone. Because the latter is the active steroid, and the conversion of prednisone to prednisolone is performed by the liver, it is possible that the de facto dosage might be diminished in proportion to the degree of liver damage (Madsbad et al. 1980).

The actual dosage of adrenocorticosteroid administered, however, was remarkably constant, with most groups using 40 mg per day (table 2).

The duration of dosage, however, is another matter. Therapy was administered for less than 1 week in one investigation (Schumaker et al. 1978) and for as long as 12 years in another (Schlicting et al. 1976) (table 2). Obviously, the gross mortality would be greater in the latter study even if ACS therapy prevented all deaths from alcoholic hepatitis. Furthermore, it is irrational to expect the ravages of a disease that develops over months or years to be reversed in days or weeks.

Several of the studies were specifically limited to patients with alcoholic hepatitis with encephalopathy (Lesesne et al. 1978; Depew et al. 1980), a subgroup with a particularly poor prognosis and one that has been reported to be especially responsive to ACS therapy (Helman et al. 1971) (table 2).

The most important disparity, however, was that the prognosis in the different groups varied tremendously. The mortality in the untreated control groups varied from 19 percent (Maddrey et al. 1978) to 100 percent (Lesesne et al. 1978). Surprisingly, there exists an impressive curvilinear relationship between the mean total serum bilirubin concentration at the time of randomization, and the percentage mortality in the untreated control groups (figure 1). The explanation for this nearly perfect correlation is not clear, but is currently under investigation.

The numbers of patients admitted to the various studies varied widely, ranging from 14 patients (Lesesne et al. 1978) to 72 (Schlicting et al. 1976) (table 2). This type of disparity raises problems in combining because the larger studies carry more weight than the smaller ones. To compensate for such differences, methods have been devised in which the number of patients studied is taken into account and weighted proportionately in the calculations (Fleiss 1973).

3. *If studies are to be combined, how should they be combined?* The summation method has already been mentioned (table 1). Using this method, the ACS therapy did not significantly improve survival (mean 5 percent).

Another simple method is the simple sign test. Whenever the survival percentage in the ACS group is greater than that in the placebo group, the study is rated a plus; when it is smaller, a minus. The more positive the difference, the more effective the treatment (figure 2). Six of the studies are plus and four are minus, and the mean difference is +5 percent. Using the simple sign test, there is no consensus.

A variant of this test is the descending sign test. In this test, the survival percentages of the placebo groups are arranged in descending order and matched with the survival percentages of the treatment groups, which are similarly arranged. The highest survival percentage of the control group is paired with the highest survival percentage of the treatment group (figure 3), the next highest with the next highest, and so forth. The sign test is then applied. In this instance, 9 of the 10 studies are plus, with a mean difference of +11 percent. This analysis favors steroid therapy.

Another variation is to arrange the survival percentage of the treatment groups in descending order and those of the placebo groups in ascending order, pairing the highest survival percentage in the treatment groups with the lowest percentage in the placebo groups (figure 4). Application of the ascending-descending sign test shows five plus and five minus, and a mean of +5 percent.

One of the most popular methods of combining ordinal data is that developed by Mantel and Haenszel (1959). In essence, a χ^2 test is weighted in favor of the investigations with the greater number of subjects. Using this test, χ^2 was 1.26 ($p > 0.30$), a statistically insignificant difference.

A variety of additional statistical techniques for combining the results of independent studies has been reviewed recently (Rosenthal 1978) (table 3). The advantages and limitations of these tests are discussed.

The heterogeneity of both the clinical material and the results in the 10 investigations of ACS treatment of alcoholic hepatitis indicate that combining these data is a bad bet.

4. *Does it help to combine data?* Sometimes, combinations of consonant but inconclusive data may be helpful. Three controlled clinical trials that compared the effect on survival of prophylactic portacaval anastomosis versus no surgery in cirrhotic patients with esophageal varices all showed statistically insignificant trends toward greater survival in the unoperated control patients (Conn et al. 1972; Jackson

et al. 1968; Resnick et al. 1969). When combined, the data achieve a consistent degree of statistical significance (figure 5). With the studies of alcoholic hepatitis, it does not appear to help.

We have focused in this presentation on the virtues of homogeneity and the vices of heterogeneity. In fact, the heterogeneity of the clinical material can be responsible, in part, for heterogeneous results. By studying groups of patients in whom the mortality is nil (figure 1), one can prove in controlled trials that no form of therapy can significantly improve survival.

It is not enough to perform controlled trials; it is important that investigations be designed to study those patients in whom benefits are not only desirable, but also achievable.

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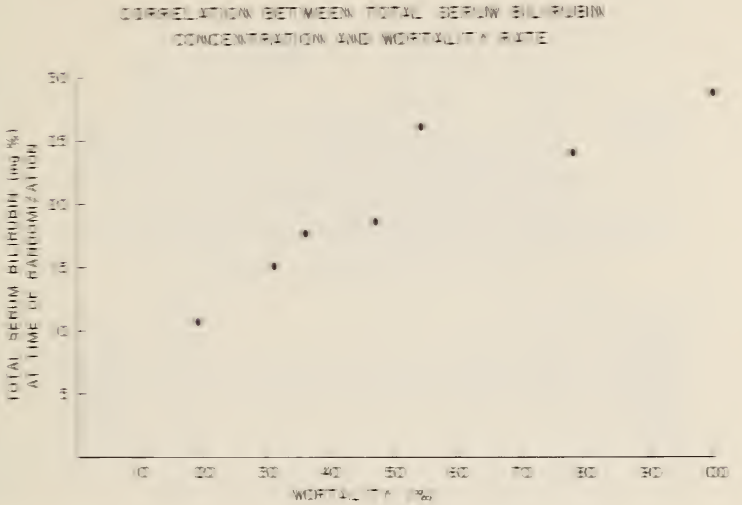
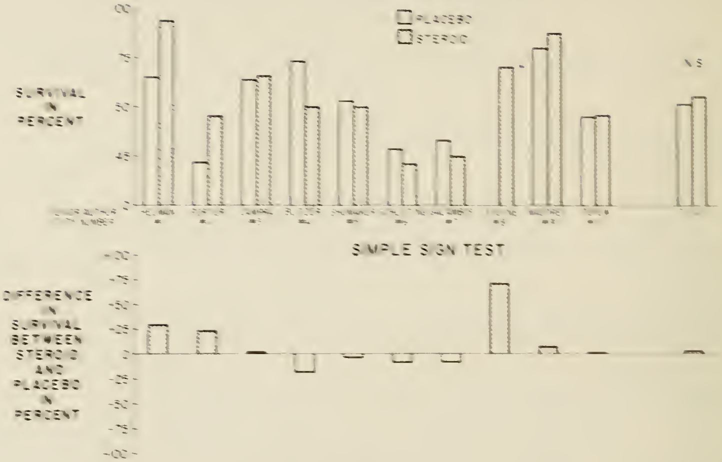
Figure 1. Correlation of Total Serum Bilirubin Concentration and Mortality in Alcoholic Hepatitis



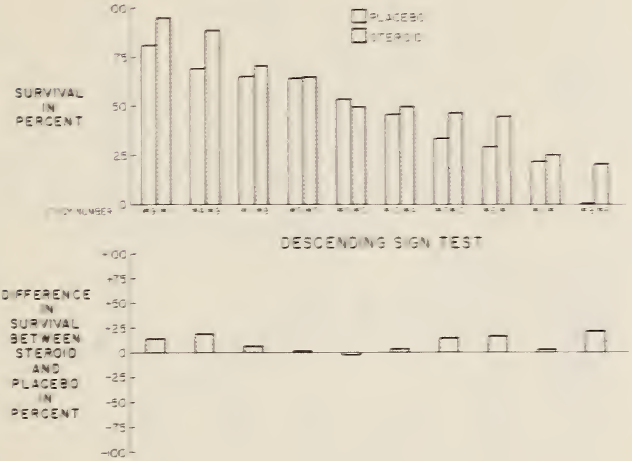
The mean total serum bilirubin at the time of randomization of the untreated control groups of 12 controlled clinical trials reported in the literature shows a positive correlation with the mortality percentage. The circles represent seven investigations in which adrenocorticosteroid therapy was evaluated (Porter et al. 1971; Campra et al. 1973; Blitzer et al. 1977; Schumaker et al. 1978; Lesesne et al. 1978; Maddrey et al. 1978; Depew et al. 1980) and five in which another therapy was investigated (Resnick et al. 1974; Nasrallah and Galambos, 1980; Baker et al. 1981; Henning, 1981; Sánchez-Tapias et al. 1981).

Figure 2. Simple Sign Test



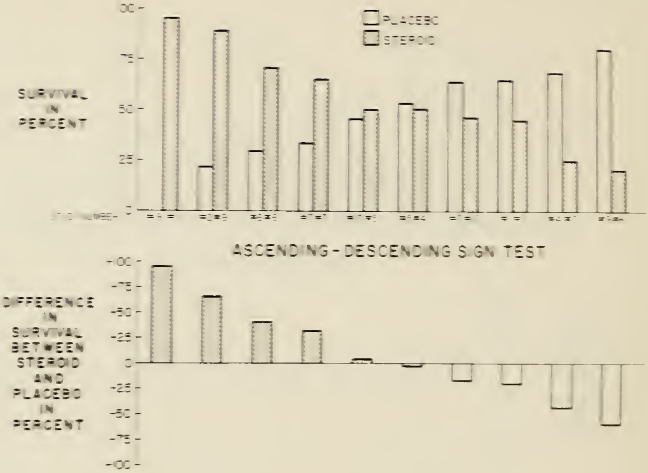
The survival percentages of the placebo and steroid groups from each of the 10 investigations are paired (upper portion). The studies are identified by name and study number beneath each pair (table 1). The difference in survival percentage (steroid − placebo) are plotted in the lower portion of the figure (positive differences above the line, negative differences below). Six of the studies showed plus signs and four minus signs.

Figure 3. Descending Sign Test



The highest survival percentage of the placebo group is paired with the highest survival percentage of the treatment group. The second highest placebo percentage is paired with the second highest steroid percentage, etc. The study numbers (table 1) are indicated below each pair (upper portion). The differences in survival percentage (treatment — placebo) are plotted as in figure 2 (lower portion). Nine of the 10 pairs showed positive signs.

Figure 4. Ascending-Descending Sign Test



The lowest survival percentage of a placebo group is paired with the highest survival percentage of a steroid group from the 10 studies. The second lowest placebo survival percentage is paired with the second highest steroid survival percentage and so forth. The studies are identified by study number (table 1) (upper portion). The differences in survival percentage are shown in the lower portion (steroid − placebo). Five of the tests showed plus signs and five minus signs.

Figure 5. Cumulative Survival in Three Controlled Clinical Trials of Prophylactic Portacaval Anastomosis (A,B,C,D)

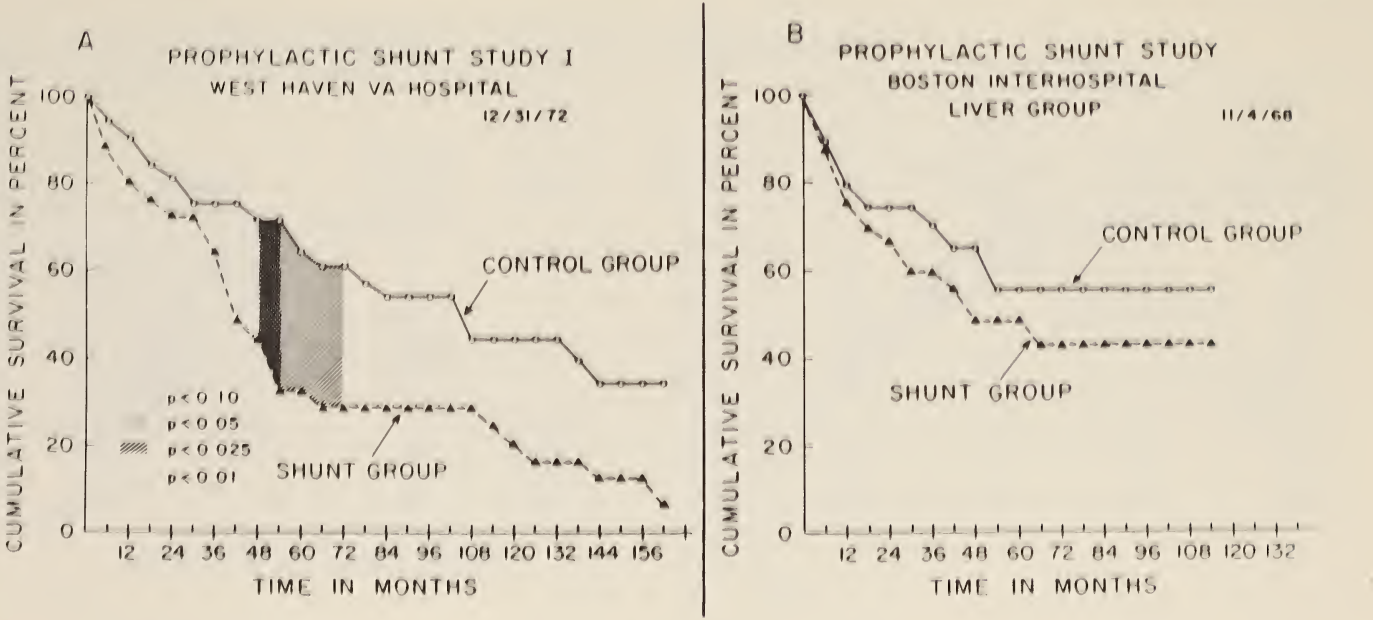
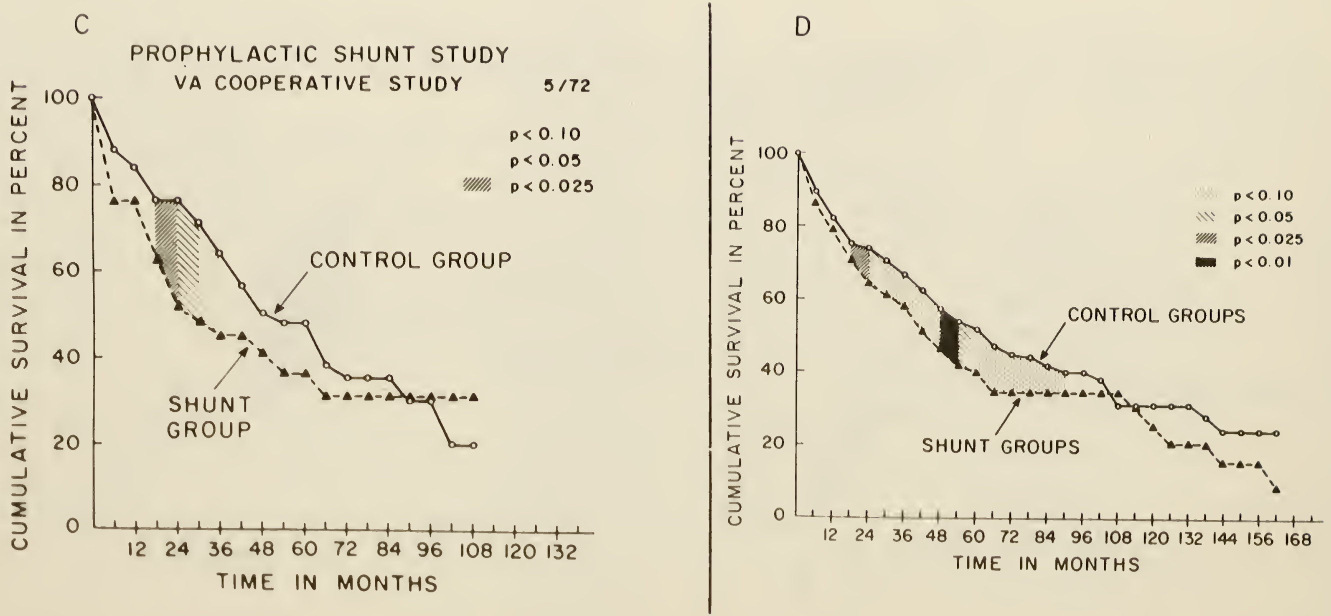


Figure 5. Cumulative Survival in Three Controlled Clinical Trials of Prophylactic Portacaval Anastomosis (A,B,C,D)—Continued



In each study a trend toward better survival in the unoperated, control groups is apparent, but the differences are only sporadically significant statistically (A, Conn et al. 1965; B, Resnick et al. 1969; C, Jackson et al. 1968). Combining these data (D) indicates that the difference in survival achieves statistical significance ($p < 0.05$) for most of the period between 18 and 60 months after randomization.

Table 1. Double Blind Controlled Studies of Steroids in Alcoholic Hepatitis

Study No.	Authors	Placebo			Steroid			Statistical Significance
		No. of Patients	No. of Deaths	Survival (percent)	No. of Patients	No. of Deaths	Survival (percent)	
1	Helman et al. (1971)	17	6	65	20	1	95	$p < .025$
2	Porter et al. (1971)	9	7	22	11	6	45	NS
3	Campra et al. (1973)	25	9	64	20	7	65	NS
4	Blitzer et al. (1977)	16	5	69	12	6	50	NS
5	Schumaker et al. (1978)	15	7	53	12	6	50	NS
6	Schlieting et al. (1976)	38	27	29	34	27	21	NS
7	Galambos (pers. comm.)	9	6	33	8	6	25	NS
8	Lesesne et al. (1978)	7	7	0	7	2	71	$p < 0.001$
9	Maddrey et al. (1978)	32	6	81	25	3	88	NS
10	Depew et al. (1980)	13	7	46	15	8	47	NS
	Total	181	87	52	164	72	57	NS

Table 2. **Comparison of Investigations of Adrenocorticosteroid Therapy in Alcoholic Hepatitis**

Author	No. of Patients	Male (percent)	Mean Age (years)	Steroid Used	Dosage (mg/d)	Duration of Therapy	Other
Helman et al. (1971)	37	32	48	Prednisolone	40	28	Liver biopsy required
Porter et al. (1971)	20	65	47	6-Methyl prednisolone	40	10	
Campra et al. (1973)	45	38	43	Prednisone	40*	21	Not double blind
Blitzer et al. (1977)	28	100	48	Prednisolone	40	14	
Schumaker et al. (1978)	27	44	45	6-Methyl prednisolone	80	4-7	Date of birth randomization
Schlicting et al. (1976)	72	86	59	Prednisone	40-20	up to 4,380	Date of birth randomization
Galambos (pers. comm.)	17	—	—	—	—	—	
Lesesne et al. (1978)	14	59	49	Prednisolone	40	30	PSE
Maddrey et al. (1978)	57	64	41	Prednisolone	40	30	
Depew et al. (1980)	28	57	49	Prednisolone	40	28	PSE

*0.5 mg/kg Body weight.

Table 3. Advantages and Limitations of Nine Methods of Combining Probabilities

Method	Advantages	Limitations	Applicable When
Adding logs	Well established	Cumulates poorly; can support opposite conclusions	*N* of studies is small (≤ 5)
Adding *ps*	Good power	Inapplicable when *N* of studies (or *ps*) is large, unless complex corrections are introduced	*N* of studies is small ($\Sigma p \leq 1.0$)
Adding *ts*	Unaffected by *N* of studies, given minimum *df* per study	Inapplicable when *ts* are based on very few *df*	Studies are not based on too few *df*
Adding *Zs*	Routinely applicable, simple	Assumes unit variance when under some conditions Type I or Type II errors may be increased	Anytime
Adding weighted *Zs*	Routinely applicable permits weighting	Assumes unit variance when under some conditions Type I or Type II errors may be increased	Whenever weighting is desired
Testing mean *p*	Simple	*N* of studies should not be less than four.	*N* of studies ≥ 4
Testing mean *Z*	No assumption of unit variance	Low power when *N* of studies is small	*N* of studies ≥ 5
Counting*	Simple and robust	Large *N* of studies is needed; may be low in power	*N* of studies is large
Blocking	Displays all means for inspection, thus facilitating search for moderator variables	Laborious when *N* is large; insufficient data may be available	*N* of studies is not too large

(From Rosenthal, R. Combining results of independent studies. *Psych.Bull.*, 85:185-193, 1978. Copyright 1978 by the American Psychological Association [Wash., DC].)

Treatment of Alcoholic Liver Disease: Colchicine*

Marcos Rojkind and David Kershenobich

Introduction

Colchicine, a naturally occurring alkaloid obtained from the plant *Colchicum autumnale L.*, has been used for many centuries in the treatment of gout without clear knowledge of its mechanism of action or the metabolites produced (Godman and Gilman 1970). At the average dose used in acute gout (1.2 to 6 mg/day), its side reactions are minimal and are related to the gastrointestinal alterations produced (nausea, vomiting, cramping, diarrhea) (Godman and Gilman 1970). Its chronic use, however, can produce azoospermia (Merlin 1972). When large doses of colchicine are given intravenously or ingested accidentally or for suicidal purposes, the drug is toxic and produces liver and bone marrow alterations (hepatic necrosis, agranulocytosis) that are directly responsible for the person's death. Several such cases have been reported in the literature (Caplan et al. 1980; Fernández et al. 1980).

Although the antimitotic activity of colchicine was discovered in 1934, it was not until 1954 that this pharmacological property of the drug was used for chromosomal studies and karyotype determination (Dustin 1963). Colchicine arrests mitosis in methaphase, allowing proper separation and counting of chromasomes. Because of this antimitotic effect, colchicine could be used as a cytostatic agent in the treatment of some malignant tumors.

The use of colchicine in the treatment of liver cirrhosis was conceived in our laboratory in 1972 and was derived from two important observations in the literature:

1. Colchicine is an antimicrotubular agent, and collagen requires the microtubular system for this transcellular movement (Diegelman and Peterkofsky 1972; Ehrlich and Bornstein 1972; Ehrlich et al. 1974).
2. Colchicine enhances the production of collagenase when added to synovial tissue cultures (Harris and Krane 1971).

Accordingly, colchicine has the two pharmacological effects needed to fight liver fibrosis. It could inhibit transport and synthesis of collagen

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NOTE: Tables appear at end of paper.

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and also destroy old tissue collagen through the induction of collagenase. This latter effect is of particular importance, since it has been demonstrated in the experimental animal with cirrhosis that active fibrogenesis and the metabolic changes that take place in the liver in direct association with the deposition of collagen all return to normal upon discontinuation of the toxic agent that produced cirrhosis (Ehrinpreis et al. 1980; Galligani et al. 1979; Rojkind and Kershenobich 1975). Therefore, if this process also occurs in alcoholic cirrhotics that withdraw from alcohol, the activity of liver fibrogenesis may be low or normal, in spite of the presence of fibrosis. In such cases, antifibrogenic drugs that only inhibit active collagen synthesis and deposition will have no beneficial effect on the fibrotic process. In this particular situation, which may be more common than believed, drugs that will enhance the capacity of the liver to remove collagen may prove to be valuable.

Many of the beneficial effects of colchicine in cirrhosis, as will be shown later, are not related to its antimicrotubular action. The improvement in liver function is independent of the degree of resorption of the preexisting scar in cirrhotic liver (Kershenobich et al. 1979; Rojkind et al. 1973). Although most of our patients show clinical and biochemical improvements, in only a few cases have we seen disappearance of liver fibrosis after 1 or more years on colchicine (Kershenobich et al. 1979; Rojkind et al. 1973). Furthermore, we still do not know whether colchicine or one of its metabolites (colchiceine) or both are responsible for the pharmacological activities that could explain the improvement in liver function observed in our animals (Rojkind and Kershenobich 1975) and in patients with liver cirrhosis (Kershenobich et al. 1979; Rojkind et al. 1973). A list of some pharmacological effects of colchicine and colchiceine is shown in table 1.

Our clinical experience with the use of colchicine is based on more than 100 patients, of whom more than half are part of a double blind randomized trial, the results of which were published after a group of 43 patients completed a minimum of a 4-year followup period (Kershenobich et al. 1979). The other group is made of the patients who did not meet the criteria to be included in the double blind trial and contains mainly two types of patients: cirrhotics with prothrombine time alterations and lack of liver biopsy at the beginning of the trial, and patients with bilirubin values above 1 mg. In this group we also selected patients that met the criteria to be included in the double blind trial, but who for personal or other reasons did not want to take the risk of belonging to the placebo group. Twenty subjects in this open group, including the patients of our original report (Rojkind et al. 1973), have already completed a minimum of 5 or a maximum of 8 years in the trial. The results obtained with both groups, randomized and nonrandomized cirrhotics, were the same, and they are summarized in table 2.

Clinical Changes

Patients who had edema and ascites at the beginning of the trial did not require as frequent treatment for fluid retention as did the placebo or control population. Furthermore, in many of those patients, after the initial months on colchicine, edema and ascites disappeared and no further treatment was needed. The episodes of bleeding and encephalopathy also decreased in number and frequency. In some patients, no further bleeding or encephalopathy have been observed. This result is in striking contrast to the placebo or control groups, since patients without bleeding or encephalopathy at the beginning of the study developed these complications during the course of the trial.

Although in many patients on colchicine the size of the spleen decreased, the presence of esophageal varices was not modified, nor was the size of the liver. No direct measurements of portal pressure were performed in these patients.

Biochemical Changes

In the open trial, which contained patients with abnormalities in bilirubin and prothrombine time, the values returned to normal after 24 months of treatment. Some patients in whom biopsy was not possible because of prothrombine time abnormalities have since had one or more liver biopsies. In 65 percent of colchicine-treated patients with bilirubin above 1 mg, this value is currently below 1 mg.

Serum albumin remained normal or increased to normal values in patients treated with colchicine. In the placebo or control groups, albumin deteriorated with time and was significantly lower than in the colchicine group. It is possible that the improvement in ascites and edema could be related in part to the improvement in serum albumin values.

The only biochemical abnormality thus far observed, secondary to colchicine treatment, is the increase in alkaline phosphatase serum levels. This enzyme is apparently derived from the liver plasma membrane (Ikehara et al. 1978; Wilfred 1977). The reason for this alteration is not known. However, recent work from our laboratory has shown that although in CCl_4-induced cirrhosis in rats, liver plasma membranes contain abnormally low concentrations of alkaline phosphatase, colchicine treatment improves this value in addition to increasing its release into the blood (García-Tsao et al. 1980).

Alcoholic cirrhotics differ in some biochemical parameters from other cirrhotic patients. It has been previously shown that alcoholic cirrhotics contain elevated serum proline values (Mata et al. 1975). Furthermore, in alcoholic patients it has been found that the procollagen (Type III) peptide reaches abnormally high values in serum (Rohde et al. 1979). In addition to these abnormalities, the alcoholic patient with cirrhosis has elevated lactic acid values in blood, and the values have a linear correlation with serum proline (Kershenobich et al. in press). Although we do not have information about the procollagen peptide values in our patients, proline and lactic acid values return to normal after 6 months to 1 year on colchicine (Kershenobich et al. in press). The mechanism for this improvement is not known; however, it is possible that colchicine improves lactic acid by modifying intermediary metabolism secondary to its action on the plasma membrane adenyl cyclase system.

Rats treated chronically with CCl_4 show increases in plasma membrane adenyl cyclase and cytosolic cAMP and a significant decrease in liver glycogen content. Colchicine-treated animals showed normal values of adenyl cyclase activity, cAMP, and liver glycogen (Mourelle et al. in press).

Histological Changes

Due to the sampling error of needle liver biopsies, and the relatively small numbers of patients so far studied, our histological observations should be taken cautiously. However, in many of our patients we have performed three or more serial biopsies, and our observations refer to those patients.

In most cases, the histology appears to remain the same after colchicine treatment; however, the lesions do not progress further. In some other patients, with slight preference for females, complete clearing of fibrosis is observed.

Survival Analysis

The 4-year survival rate in the colchicine-treated patients (double blind) and 5-year survival rate in the open trial is over 75 percent. In the placebo group, however, the survival rate is only 50 percent. This prolonged survival in the colchicine-treated cirrhotics is accompanied also by an improvement in quality of life.

In summary, colchicine improves liver function of cirrhotic patients. In some cases, modifications in liver fibrosis are also observed. These changes are independent of whether the patient continues to ingest alcohol. The mechanism of action of colchicine is not primarily antifibrogenic but appears to be related to the modifications in membrane function induced by colchicine. The latter changes are in plasma membrane of hepatocytes but also occur in inflammatory cells (see table 1), thus modifying the inflammatory and perhaps immunological reactivities of the host.

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Table 1. **Some Pharmacological Effects of Colchicine and Colchiceine**

1. Stabilizes lyosomal membranes
2. Inhibits mobilization and extracellular release of enzyme granules in human leukocytes during phagocytosis
3. Inhibits histamine liberation from leukocytes (high dose)
4. Inhibits in vitro liberation of kinin release from plasma (high dose)
5. Inhibits mobilization, adherence, and chemotaxis of neutrophils
6. Inhibits neutrophil-mediated antibody-dependent cytotoxicity
7. Modifies cAMP levels in leukocytes
8. Modifies the adenylate cyclase system in the liver (enzyme and cAMP)
9. Increases plasma levels of alkaline phosphatase
10. Increases liver glycogen (in cirrhotic animals)
11. Lowers plasma proline and blood lactate in alcoholic cirrhotic patients
12. Inhibits turnover of phosphatidyl inositol in lymphocytes
13. Decreases proteinuria in nephrotic syndrome
14. Increases skin darkening induced by melanocyte-stimulating hormone
15. Inhibits amyloid formation
16. Inhibits transcellular movement of export proteins including collagen
17. Induces collagenase production in synovial tissue
18. Inhibits collagenase activation induced by proteases
19. Colchiceine protects reduced glutathione from oxidation by O_2
20. Colchiceine protects SH-containing enzymes
21. Colchiceine binds to liver plasma membrane and gangliosides GT1, GDa, and GM1

Table 2. **Improvement in Some Clinical and Biochemical Manifestations in Cirrhotic Patients Treated with Colchicine**[a]

Abnormality	Approximate Time Needed for Improvement[b] (months)	Improvement[c] (percent)
Encephalopathy	18-24	60
G.I. Bleeding	18-24	60
Fluid Retention (Ascites and edema)	12-18	73
Prolonged Prothrombine Time	24-36	55
Bilirubin > 1 mg	6-12	65
Hypoalbuminemia	6-12	70
Increase in Alkaline Phosphatase[d]	6-12	90

[a] Summary of data obtained from 42 surviving patients in the double blind trial (22 patients) (Kershenobich et al. 1979) and in an open trial (20 patients) (to be published).

[b] Average time required for at least 50 percent of the patients to show improvement of a particular abnormality.

[c] Percentage of patients who showed improvement of a particular abnormality.

[d] Most of the patients on colchicine showed increases in serum alkaline phosphatase after 6 months. Values continued to increase thereafter.

Penicillamine in Alcoholic Liver Disease*

Irmin Sternlieb

D-penicillamine was first introduced 24 years ago as a chelating agent for copper (Walshe 1956). Since then it has proved to be remarkably successful in controlling and even reversing the ravages of copper in the liver and central nervous system of patients with Wilson's disease (Sternlieb and Scheinberg 1979; Walshe 1976). In 1963, D-penicillamine was shown to be effective in treating patients with cystinuria by reducing cystine excretion (Crawhall et al. 1963, 1979). About one-third of patients with rheumatoid arthritis benefit from treatment with D-penicillamine, although the mechanism by which the drug is effective is still a subject of debate (Jaffe 1979). These and other pharmacologic effects of this fascinating drug are listed in table 1.

Interest in the use of penicillamine for non-Wilsonian liver diseases stems from its ability to inhibit collagen biosynthesis cross-links by forming thiazolidine rings with lysyl-derived aldehydes (Siegel 1977). This effect can be observed in both in vitro cultures (Uitto 1969) and intact rats kept on choline-deficient diets (Perings et al. 1973). In a clinical trial Mezey et al. (1979) found that penicillamine depressed elevated levels of hepatic collagen proline hydroxylase in three patients with alcoholic hepatitis, while five similarly tested placebo-treated patients showed elevations of the same enzyme in followup biopsy specimens of liver. Obviously, synthesis of collagen continued in the placebo-treated group but not in the D-penicillamine-treated patients. In the course of the same study, Mezey also obtained evidence for enhanced degradation of collagen in penicillamine-treated patients, further suggesting that penicillamine might be useful for the prevention of collagen deposits that accompany alcoholic liver disease.

By mechanisms unrelated to those discussed so far, D-penicillamine can be shown to divert part of acetaldehyde generated during ethanol metabolism in rats by forming thiazolidine carboxylic acid, which is excreted in the urine (Nagasawa et al. 1977). Under certain experimental conditions this drug stabilizes lysosomal membranes in

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Note: Tables appear at end of paper.

* This work was supported in part by grants from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM 17702) and the Foundation for the Study of Wilson's Disease, Inc.

vitro and in vivo, thus exerting a possible protective effect on liver cells (Carevic 1980). Furthermore, it has an anti-inflammatory action, it interferes with polymorphonuclear leukocyte chemotaxis, and it inhibits human Helper T-cell function in vitro (Lipsky and Ziff 1980). With such an impressive list of credentials, it was reasonable to assume that penicillamine might affect the course of alcoholic liver injury.

There are only a few published studies on the effects of penicillamine therapy in alcoholic liver injury. In a few patients with decompensated alcoholic cirrhosis treated by Seelig et al. (1980), general improvement and a possible reduction of the amount of collagen in liver biopsy specimens was noted while the patients were kept on a combined regimen of penicillamine and large doses of vitamins. However, in a controlled trial in patients with acute alcoholic heptitis, Resnick et al. (1974) did not find an increased survival in the penicillamine-treated cohort, although the drug seemed to have a beneficial effect on the severity of the histologic lesions observed in serial liver biopsy specimens. This last observation is somewhat surprising, since we have failed to observe significant effects of D-penicillamine on the amounts of fibrous tissue in cirrhotic livers of patients with Wilson's disease; nevertheless hepatocellular function was clearly improved in the majority of these subjects (table 2). Consequently, we have to conclude that at present there is insufficient evidence that penicillamine can play a role in the treatment of liver diseases other than those associated with excessive accumulations of copper.

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Table 1. Effects of Penicillamine

- Chelates heavy metals: Cu, Pb, Au, Hg
- Interferes with cross-linking of collagen and elastin
- Enhances excretion of acetaldehyde
- Stabilizes lysosomes (in vitro and in vivo)
- Has anti-inflammatory action
- Interferes with polymorphonuclear chemotaxis
- Inhibits T-cell lymphocyte function
- Dissociates macroglobulins
- Affects cysteine and cystine metabolism

Table 2. Effects of Some Clinical and Laboratory Indicators of Liver Dysfunction After 1 Year of D-Penicillamine Treatment of Patients with Wilson's Disease

Effect	No. of Patients	No. of Patients Showing Improvement
Jaundice, edema ascites	13	9
Hypoalbuminemia	16	13
Elevated aminotransferases	32	22
Hypoprothrombinemia	14	8

The Interrelationship of Nutrition and Alcoholic Liver Injury*

Michael F. Sorrell and Ruth M. Jacobs

Introduction

The place of altered nutrition in the pathogenesis of alcoholic liver injury has been controversial. Much of the controversy has been rooted in earlier concepts that proper nutrition alone would prevent alcohol-induced liver injury. This perspective shifted to the view that ethanol functioned as a direct hepatotoxin with no significant role for nutrition in the causal relationship of alcoholic liver disease. Recently, a shift toward some middle ground is evident. This overview will examine the evidence primarily as it exists in man and nonhuman primates.

Alcohol and Nutrition

Alcoholic beverages contain carbohydrates, trace elements, and a few water soluble vitamins (Leake and Silverman 1974). However, they have little nutritive value aside from providing 7.1 kilocalories per gram of ethanol.

In general, alcoholics derive over 50 percent of their total calories in the form of alcoholic beverages and often consume total calories in excess of that required for maintenance (Ristic et al. 1977). Ethanol metabolism is known to supply less energy than comparable carbohydrate metabolism. Pirola (1972) demonstrated that when 50 percent of total caloric intake was supplied by isocaloric substitution of ethanol for carbohydrate, significant weight loss ensued. Trémoliéres and Carré (1961) demonstrated increased oxygen consumption in normal subjects given ethanol. This effect was greater in the chronic alcoholic. Subsequently, other investigators (Bernstein et al. 1973; Israel et al. 1979) have reported that livers harvested from chronically ethanol-fed rats exhibited increased oxygen consumption, resulting in a hypermetabolic state. The increased oxygen consumption was thought to be the cause of centrilobular necrosis observed in chronically ethanol-fed animals that were also subjected to reduced

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oxygen tensions. However, other investigators have failed to induce a hypermetabolic state by chronic ethanol feeding (Gordon 1978).

The actual incidence of malnutrition in alcoholics is unknown. Statistics vary depending on the population, diet, and income of the group studied. Nutritional assessment of 34 alcoholic patients with adequate financial resources did not detect evidence of malnutrition (Neville et al. 1968). In another study, only 12 percent of middle-class alcoholic patients admitted to the Mayo Clinic weighed less than their ideal body weight (Hurt et al. 1979).

However, evidence of multiple vitamin deficiencies, protein malnutrition, and poor dietary intake composition is often found in derelict alcoholics. Protein calories were less than 6 percent and ethanol calories more than 50 percent of the daily intake in a large series of patients admitted to the Boston Veterans Administration Hospital (Patek et al. 1975). Mezey and Faillace (1971) found that 56 alcoholics consumed less than one meal daily for the 10-day period preceding hospital admission.

Decreased levels of water-soluble vitamins are often found in chronic alcoholics, whereas circulating levels of fat soluble vitamins are usually normal unless the patient has advanced liver disease. Leevy et al. (1965) obtained a history of grossly substandard diets in 64 percent of 172 patients with a history of chronic alcoholism. Circulating levels of two or more water-soluble vitamins were significantly reduced in 32 percent of patients with normal livers, in 44 percent of those with fatty livers, and in 49 percent of those with cirrhosis. Folate was the most common vitamin deficiency described, with decreased serum folate levels found in 47 percent of all cirrhotics. Inadequate dietary intake is an important factor affecting the overall nutritional status of the alcoholic. In addition, ethanol directly alters absorption, storage, activation, and catabolism of many important nutrients.

Clinical Studies in Man

Because early descriptions of alcoholic liver injury were primarily concerned with skid row alcoholics with concomitant malnutrition, the popular etiologic construct evolved that poor nutrition was the culprit rather than alcohol itself. The early observations of Best and coworkers (1949) that growing rats fed diets deficient in choline and methionine plus 15 percent alcohol in the drinking water developed a fatty liver correlated with this view. The concept that emerged of an alcohol-induced relative lipotrope deficiency was then extended to humans rather uncritically. Subsequently, several studies in human volunteers fed ethanol in which larger supplemental amounts of choline and lipotropes were given failed either to prevent the development of a fatty liver or to accelerate recovery from alcoholic liver injury (Leevy 1962; Olson 1968). Klatskin et al. (1954) administered alcohol to rats and demonstrated increased choline requirements, and others have shown that choline increases choline oxidation in the perfused liver (Barak et al. 1973). However, a comparable choline deficiency state has not been produced in humans. Therefore, choline deficiency per se seems to be primarily a rodent disease. Rodents possess high levels of choline oxidase, whereas primates and humans have low levels resulting in lower dietary requirements for choline.

Based on early animal data and the nutritional observations on derelict alcoholics previously discussed, preliminary uncontrolled nutritional treatment trials were reported (Patek and Post 1941; Patek et al. 1948) and supported the view that nutritional therapy was beneficial. Subsequently, hospitalized recovering alcoholics with liver disease were given similar amounts of alcohol but diets that varied in amount of protein (Erenoglu et al. 1964). Serial liver biopsies and liver function tests were performed. The patients ingesting the high protein diet showed steady clinical improvement that was not mirrored by changes in hepatic histology. The patients fed the low-protein diet were believed to show no change or slight deterioration in all parameters measured. Other investigators in related studies confirmed the observation that simultaneous administration of modest amounts of alcohol did not inhibit recovery from alcoholic liver injury (Summerskill et al. 1957; Volwiler et al. 1948).

Additional evidence supporting altered nutrition as a causal factor in the production of liver disease has developed with the reports of severe liver disease resembling that seen in alcoholics but associated with the various operations designed to treat morbid obesity. Small bowel bypass with a resultant that 90 percent jejunoileal discontinuity has resulted in the production of the entire spectrum of liver disease ranging from fatty liver to alcoholic hepatitis and in a few cases to cirrhosis (Peters et al. 1975). A histologic picture indistinguishable from alcholic hepatitis has also been reported after massive small bowel resection (Peura et al. 1980) and gastric bypass for obesity (Galambos, personal communication). In addition, reversal of the small-bowel-bypass-induced liver disease has been reported by either restoration of intestinal continuity (Soyer et al. 1976) or by employment of total parenteral nutrition (Baker et al. 1979). Recently, Nasrallah and Galambos (1980) have reported on a randomized clinical trial of intravenous amino acid therapy in patients with severe alcoholic hepatitis. Peripheral intravenous alimentation for 4 weeks was associated with an increased survival rate, improved serum bilirubin, and increased albumin concentrations when compared to the control group. These studies lend support to the view that nutrition may be an important factor in alcoholics but are far from conclusive in proving causation.

Conversely, an immense body of information of both a direct experimental as well as an epidemiologic nature suggests a role for ethanol as a direct hepatotoxin in humans. Lelbach (1976) has shown that the incidence of alcoholic cirrhosis is directly proportional to the amount and duration of ethanol consumed per capita. In a series of elegant experiments, Lieber and colleagues (Lieber et al. 1963, 1965; Lane and Lieber 1966) gave alcohol to alcoholic volunteers with recent histories of abstention. All subjects given alcohol developed a fatty liver, as determined by histologic and biochemical means, despite an adequate dietary intake. Subsequently, these findings were confirmed in nonalcoholic volunteers (Rubin and Lieber 1968).

Clearly, these studies demonstrate that alcohol functions as a direct hepatotoxin but do not exclude the possible unfavorable interaction of ethanol with a number of nutritional factors. These factors could include calorie/nutrient imbalance; increased nutrient requirements; altered absorption, storage, release, and degradation of various nutrients; and perhaps the permissive and synergistic role of poor nutrition superimposed on alcohol ingestion.

Evidence in Experimental Animals

Rodents

A vast literature has emerged that is concerned with the role of alcohol and nutrition in experimental animals. For various reasons, including ease of handling, cost, and high lipotrope requirements, the growing rat became the animal of choice. As previously mentioned, much of the early enthusiasm for a nutritional role in alcoholic liver injury was sparked by the observation that nutritional fatty liver could be produced in the rat given a diet marginal in lipotrope activity and 15 percent alcohol in the drinking water (Best et al. 1949). However, alcohol alone in drinking water without a lipotrope-deficient diet failed to produce a significant fatty liver. This result was primarily due to the rodents' aversion to ethanol when administered in amounts totaling more than 20 percent of the caloric intake. This dose of ethanol results in negligible blood alcohol levels in the rat. Lieber et al. (1965) developed an all-liquid nutritionally adequate diet that provided 36 percent of the total calories as alcohol.

This diet uniformly produced a fatty liver and provided a convenient way to study the metabolic effects of alcohol. This approach, which demonstrated the direct hepatotoxicity of ethanol, was contested by others using a different dietary model. These investigators (Porta et al. 1972), administering ethanol in sweetened water and a basal highly fortified "super diet," were able to prevent the ethanol-induced fatty liver. In addition, the regression of a lipotrope-deficiency-induced rodent cirrhosis continued, despite concomitant ethanol administration. These studies were interpreted as supporting the view that ethanol exerted its hepatotoxicity by altering nutritional requirements. However, lipotrope requirements are vastly different for the rodent, and the amount of alcohol consumed as well as the duration of consumption does not approach that seen in the usual alcoholic. Most important, in the absence of a lipotrope deficiency, neither cirrhosis nor alcoholic hepatitis has been unequivocally produced in the rodent by alcohol.

Primates

In an attempt to overcome the limitations inherent to the rodent-alcohol model, Lieber and colleages turned to the primate, a species more closely related phylogenetically to man. It was felt that primates were metabolically more similar to humans (including their choline requirements), were more long lived, and were capable of ingesting up to 50 percent of their total caloric requirement as alcohol. Adapting the Lieber-DeCarli diet to the baboon's nutritional requirements allowed chronic delivery of up to 50 percent of the baboon's total calories as alcohol (Lieber and DeCarli 1974). Initially, the entire spectrum of liver disease, including alcoholic hepatitis, was described (Rubin and Lieber 1973), but subsequently it has been reported that the major lesion produced is cirrhosis, and alcoholic hepatitis does not occur (Popper and Lieber 1980). These data have been interpreted as supporting the concept of direct ethanol hepatotoxicity because cirrhosis developed during administration of a nutritionally adequate diet.

The production of cirrhosis in an animal model by chronic ethanol administration represents a major experimental accomplishment and provides an animal model that more closely mimics human disease. This model clearly demonstrates a direct role of ethanol per se in the development of liver disease. A few reservations remain as to the ultimate role of nutrition in the overall picture. The ethanol-fed baboons do not gain weight like their pair-fed controls (Lieber and DeCarli 1974). Ideally, comparison to ad-lib-fed baboon controls would provide a better overall estimate as to the adequacy of total caloric intake in the experimental groups. The choline requirement of the baboon is unknown but thought to be similar to humans. Rogers et al. (1979) have questioned the adequacy of the choline supplementation in the Lieber-DeCarli diet as modified for the baboon, although the diet contains enough choline to satisfy current primate standards (Foy et al. 1964). Even though an adequate diet has been ingested by the baboons, malabsorption of various nutrients can occur, as has been documented in other species. Blood vitamin levels have been determined in chronically ethanol-fed baboons, and circulating levels of all vitamins have been normal (Lieber, personal communication). Other factors to be considered include increased nutrient catabolism and decreased storage. Clearly, a more informed estimate of the role of nutrients in the baboon model awaits publication of the detailed nutritional status of the experimental groups. The data, although impressive in their indictment of ethanol as a direct hepatotoxin, do not exclude a concomitant permissive role of altered nutrition in the overall pathologic process.

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Hepatic Vitamin A Depletion After Chronic Ethanol Consumption

Mayumi Sato and Charles S. Lieber

Vitamin A deficiency has been of clinical interest in alcoholics, since some evidences, such as nightblindness, have been reported (Patek and Haig 1939). Recent studies have shown that plasma vitamin A and retinol-binding protein (RBP) levels are decreased in alcoholic patients (McClain et al. 1979; Smith et al. 1975). However, these patients commonly have poor dietary intake (McClain et al. 1979; Patek et al. 1975) as well as hepatic cirrhosis and zinc deficiency (McClain et al. 1979; Smith et al. 1975), which have been shown to be associated with vitamin A deficiency (Smith et al. 1971, 1973). More recently, it has been reported that alcoholism is not necessarily accompanied by impaired night vision (Campbell et al. 1981). Therefore, we wondered whether alcohol consumption directly affects the status of vitamin A and RBP, or whether the effect might result from malnutrition.

Thirty adolescent baboons were pair-fed a nutritionally adequate liquid diet containing 50 percent of total calories as either ethanol or isocaloric carbohydrate for 4 to 84 months as previously described (Lieber and DeCarli 1974). After 4 months, ethanol-fed baboons showed a fatty liver with a 59 percent decrease in hepatic vitamin A concentration compared to their pair-fed controls, as shown in figure 1. After 12 to 84 months, seven ethanol-fed baboons developed fatty liver with a 67 percent decrease in hepatic vitamin A levels, whereas four ethanol-fed animals progressed to fibrosis or cirrhosis and their hepatic vitamin A levels were decreased to only 5 percent of their pair-fed controls (figure 1). On the other hand, plasma vitamin A and RBP were increased in ethanol-fed baboons with fatty liver but were not different in fibrotic or cirrhotic stages.

Similarly, in rats pair-fed a nutritionally adequate liquid diet containing 36 percent of total calories as either ethanol or isocaloric carbohydrate (Lieber and DeCarli 1970), hepatic vitamin A was decreased after 3 weeks of ethanol feeding and continued to decrease up to 9 weeks (Sato and Lieber in press) (table 1). In contrast to the liver, vitamin A content in the kidney and testis was increased two- to threefold in ethanol-fed rats after 9 weeks. Serum vitamin A and RBP

Note: Figure and tables appear at end of paper.

levels were not significantly changed throughout the observed period. Thus, even in two nutritionally adequate animal models, hepatic vitamin A was decreased after chronic ethanol consumption, and therefore the deficiency did not seem to be due to malnutrition.

We next wondered whether this effect results from malabsorption. To study this possibility, rats were pair-fed either ethanol or a control diet that was virtually devoid of vitamin A to eliminate the effect of intestinal absorption of vitamin A. Hepatic vitamin A was again found to be much lower in ethanol-fed rats than in controls after 6 weeks, and the difference in hepatic vitamin A between these two groups was much greater than could be accounted for by the total dietary vitamin A intake during the 6 weeks. Therefore, malabsorption did not appear to explain the depletion of hepatic vitamin A after chronic ethanol consumption.

To investigate possible mechanisms other than malnutrition and malabsorption, catabolic pathways of vitamin A in the liver were studied. It has been reported that hepatic microsomal cytochrome P-450 dependent metabolism of retinoic acid (a physiological metabolite of vitamin A) is postulated to represent one of the degradative pathways for the elimination of retinoic acid from the body (Roberts and Frolik 1979). Ethanol-fed rats showed enhanced microsomal cytochrome P-450 dependent retinoic acid metabolism (50 percent, $p < 0.01$) accompanied by increased hepatic microsomal P-450 content (34 percent, $p < 0.005$). On the other hand, β-glucuronidation of retinoic acid was not significantly affected by chronic ethanol feeding. To test the in vivo metabolism of retinoic acid, rats pair-fed either an ethanol or a control diet were injected ^{3}H-retinoic acid intravenously. Urinary excretion of radioactivity was significantly increased in ethanol-fed rats compared to control rats (table 2), wheras biliary excretion was not different (table 3). Thus, chronic ethanol consumption resulted in an increased microsomal cytochrome P-450 dependent metabolism of retinoic acid, which may accelerate the catabolism of vitamin A in the liver.

In conclusion, chronic ethanol consumption decreases hepatic vitamin A storage, at least in part, through some mechanisms other than malnutrition and malabsorption. Hepatic microsomal cytochrome P-450 dependent metabolism of retinoic acid is increased after chronic ethanol consumption, which may in turn enhance the catabolism of vitamin A in the liver. Thus, even in the absence of severe hepatic damage such as cirrhosis, alcoholics are prone to develop low hepatic vitamin A levels.

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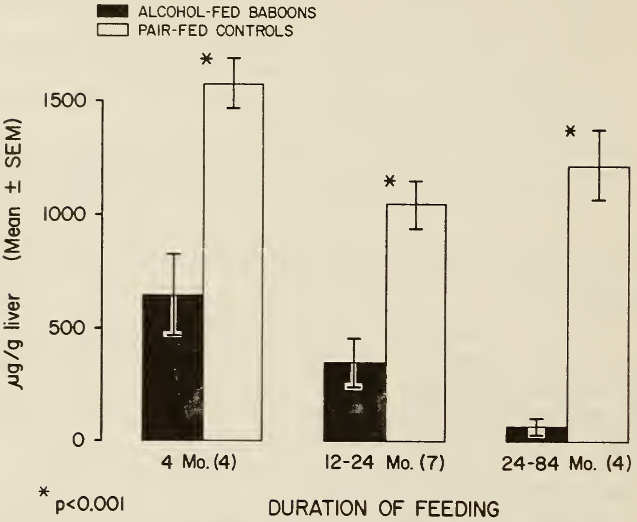
Figure 1. Effect of Chronic Ethanol Feeding on Hepatic Vitamin A Levels in Baboons



Baboons were pair-fed either ethanol or control diet and fasted for 12 hours (ethanol was withdrawn for 20 hours) before sampling. Number of pairs is shown in parentheses. Animals fed ethanol for 4 to 24 months showed hepatic steatosis, whereas fibrosis or cirrhosis was produced in the 24- to 84-months groups. (From Sato, M., and Lieber, C.S. Hepatic vitamin A depletion after chronic ethanol consumption in baboons and rats. *J Nutr*, in press. Copyright 1981 by the American Institute of Nutrition [Beth., MD].)

Table 1. Effect of Chronic Ethanol Feeding on Hepatic Vitamin A in Male Rats

Duration of feeding (No. of pairs)	Hepatic Vitamin A			
	Concentration (μg/g liver)		Total Content (μg/total liver)	
	Ethanol-fed Rats	Control Rats	Ethanol-fed Rats	Control Rats
0 weeks (6)	147.2±8.6		835.4±38.9	
3 weeks (10)	69.7±7.1*	120.0±18.2	588.0±73.8**	1196.1±115.3
4-6 weeks (10)	52.9±4.4**	131.6± 8.6	571.4±24.6**	1315.6± 62.3
7-9 weeks (3)	38.4±1.0**	138.1± 9.7	501.4±56.3**	1500.1±177.6

Note: Rats weighing 130 to 150 g were pair-fed either ethanol or control diet and fasted overnight before sacrifice. All values are expressed as mean ± SEM.

*Significantly different from control rats of the same duration of feeding ($p<0.025$).

**Significantly different from control rats of the same duration of feeding ($p<0.001$).

(From Sato, M., and Lieber, C. S. Hepatic vitamin A depletion after chronic ethanol consumption in baboons and rats. *J. Nutr*, in press, Copyright 1981 by The American Institute of Nutrition [Beth., MD].)

Table 2. Effect of Chronic Ethanol Feeding on Urinary Excretion of Radioactive Metabolites of Rats Given ^{3}H-Retinoic Acid Intravenously

Treatment (No. of rats)	Urinary Excretion (percentage of injected dose)	
	0-6 Hr	0-24 Hr
Ethanol-fed rats (5)	10.27±0.84	17.16±0.81
Control rats (5)	7.11±0.34	13.00±0.67
p	0.01	0.001

Note: Rats were pair-fed either ethanol or control diet for 4 to 6 weeks. After an overnight fast, rats were given 11,12-^{3}H-retinoic acid (1.43μCi, 54pmol/100g BW) intravenously. All values are expressed as mean ± SEM.

Table 3. Effect of Chronic Ethanol Feeding on Biliary Excretion of Radioactive Metabolites of Rats Given ^{3}H-Retinoic Acid Intravenously

		Biliary Excretion of Radioactivity	
Treatment (No. of rats)	Bile Flow (μl/min/100g BW)	Total Excretion (percent of dose/6hr)	Concentration (percent of dose/ml)
Ethanol-fed rats (5)	4.70±0.59	32.5±4.1	7.73±1.09
Control rats (5)	3.58±0.40	33.9±1.2	9.54±0.76
p	N.S.	N.S.	N.S.

Note: Rats were pair-fed either ethanol or control diet for 4 to 6 weeks and fasted overnight. After the bile duct cannulation was performed, rats were given 11,12-^{3}H-retinoic acid (0.26μCi, 10pmol/100g BW) intravenously. The data presented are average of 6-hour period and expressed as mean ± SEM.

SESSION XI: PATHOGENESIS AND TREATMENT OF ENDOCRINE ABNORMALITIES

Introduction to Discussion on Pathogenesis and Treatment of Endocrine Abnormalities

Gary G. Gordon

Abstract

The observations that alcoholic liver disease, particularly cirrhosis, causes hypogonadism and feminization were made almost 60 years ago. It has been medical dogma that these changes reflected hormonal aberrations secondary to the liver disease. Only in the last 5 years has it been documented that alcohol use, without the supervention of the cirrhosis, or indeed of any significant hepatic pathology, can produce marked changes in the blood levels of androgens and estrogens in the experimental animal and man. The alterations in blood androgens reflect toxic effects of alcohol not only in the hypothalamic-pituitary axis but also in the gonad. There is also considerable evidence that alcohol can alter the action of steroid hormones not only by affecting the secretion rate but also by changing various parameters of the kinetics of hormone metabolism (i.e. factors such as hormonal clearance and conversions) as a result of alterations in protein binding factors in blood or tissue, or changes in activity of enzyme that are rate limiting for steroidogenesis or conversion of prehormone to their active form. Finally alcohol can alter the tissue response to hormones by altering the function of tissue receptors for the hormone. We have termed these blood effects of alcohol both within the confines of the endocrine axis and in the periphery the "triple effect" of alcohol.

Ethanol-Induced Alterations in Reproductive Hormones in the Male: Factors Mediating These Effects and Their Biological Significance

Theodore J. Cicero

Abstract

This paper presents the results of several studies designed to provide answers to two important questions: First, is ethanol a direct gonadal toxin or must it be metabolized to exert its effects? Second, do ethanol-induced alterations in reproductive hormones participate in the acute and chronic biological effects of ethanol? A review of the literature, and the results described in this paper support two general conclusions. First, ethanol *per se* does not appear to directly inhibit gonadotropin-stimulated testicular steroidogenesis in the male under *in vivo* or *in vitro* conditions; rather it appears that it must be metabolized to exert its effects. Second, it appears that drug-induced changes in serum testosterone levels may be involved in the development of metabolic tolerance to ethanol and the cross-tolerance observed between ethanol and other sedative-hypnotic drugs.

Factors That Contribute to the Feminization of Chronic Alcoholic Men: New Insights

D. H. Van Thiel, P. K. Eagon, J. S. Gavaler,
and L. E. Porter

Abstract

This paper reports on an animal research project designed to elucidate the mechanisms responsible for feminization in chronic alcoholic men. The paper discusses previous research that failed to explain how cirrhotic men can be grossly feminized if their plasma estrogen levels are either normal or only minimally increased, and various hypotheses. They report that their research findings suggest that male alcohol-fed rats and presumably humans are supersensitive to estrogenic stimulation as the result of a dual abnormality in cystosolic estrogen binding proteins. The reason for this remains to be determined and many other questions must be addressed by future research, they conclude.

SESSION XII: TREATMENT APPROACHES FOR ALCOHOLISM

Introduction to Discussion on Treatment Approaches for Alcoholism

Peter E. Stokes

Abstract

The author points to the need for prevention of excessive alcohol consumption and its medical complications, and notes the limitations of animal models in understanding alcoholism. Biological treatment of alcoholism will increase in importance, he predicts, as knowledge is developed about imbalances in central nervous system (CNS) neuronal networks. The author highlights findings reported in subsequent session papers.

Lithium Treatment in Alcoholism

Laure B. Branchey

Abstract

This paper reviews two well controlled studies of the effectiveness of lithium in the treatment of alcoholism. The author concludes that current data cannot justify the widespread use of lithium, even among depressed patients, and calls for future studies that clearly define outcome measures and include both nondepressed patients and alcoholic depressed patients with primary and secondary affective disorders.

Disulfiram for Alcoholism Treatment

Richard K. Fuller

Abstract

This paper discusses studies on the efficacy of disulfiram in alcoholism treatment and concludes that almost all had significant design problems. The author reports on a controlled, blind prospective study to evaluate disulfiram; the study was conducted at the Cleveland VA Medical Center and involved 128 men. The study concludes that the implied threat of the disulfiram-ethanol reaction is an important factor in preventing drinking in those who remain abstinent; that disulfiram and counseling were more effective than counseling alone; and that compliance to the drug regimen was associated with abstinence.

Treatment Approaches for Alcoholism

Enoch Gordis and George Sereny

Abstract

The authors discuss problems related to outcome measures in alcoholism treatment, noting that all studies rely on patient self-reports and there is no biochemical measure of cumulative alcohol exposure. They question the safety of controlled drinking and suggest measures of liver function and hematological function along with measures of social function. The paper notes that it has not been possible to compare effects of treatment with no treatment. The authors suggest that the most serious flaw in outcome reports is the exclusion of patients who drop out of treatment; inadequate followup interval is another frequent flaw, as is the failure to randomize and use blind design. The paper examines controlled drinking studies, concluding that the only justifiable therapeutic recommendation at present is total abstinence, but advising an open minded approach in examining future controlled drinking studies.

CONCLUSION

Disease Control in Alcoholism: A Focused Public Health Strategy

Charles S. Lieber

In the field of alcoholism, policy makers have been sharply divided between two camps: on the one side are the advocates of a conventional public health approach against alcoholism through control of the offending agent, namely alcohol (which they blame for the damages of alcoholism), on the other side are those who espouse the thesis that it is the minority of drinkers who cannot handle alcohol that are at the core of the alcoholism problem. The first view (incriminating alcohol) prevailed with the temperance movement in the 19th and the beginning of the 20th century, culminating in prohibition. The repeal of prohibition was associated with a sharp swing towards the opposite concept, namely that the alcoholic has an innate incapacity to handle alcohol and that consequently, to control alcoholism, one has to understand and treat this special subgroup. This latter view currently permeates our society and prevails in movements such as Alcoholics Anonymous, volunteer organizations such as the National Council on Alcoholism and is consonant with the prevailing view of the alcohol industry. Organizations supporting this view have been successful in gaining recognition for the alcoholism problem and in securing modest albeit significant support for alcoholism research. In recent years the amalgamation of "pro-host" forces has been prevailing in our society and attempts at reviving some public health approach to alcoholism have not been very successful. For instance, recent efforts by the National Institute on Alcohol Abuse and Alcoholism at "stabiling" alcohol consumption in this country have been branded as "neoprohibitionist" and the approach was not successfully implemented. In general, the alcoholism field has suffered very much from this polarization between conventional public health advocates who are identified with supporters of consumption control measures and those who see the problem as a disease limited to predisposed individuals. One of the reasons for the appallingly low alcoholism research budgets in this country, exemplified by the astonishingly low NIAAA research support (Lieber, 1981), may well derive in part from this lack of consensus on where to place the emphasis of alcoholism research. For those individuals who come to the problem through a public health perspective, limiting the exposure through control of availability of alcoholic beverages appears the natural approach (in the absence of any reasonable possibility of immunizing against the chemical agent). For those convinced that the alcoholic will drink excessively regardless of such control measures, consumption control appears as a futile measure which encroaches on the freedom of action of the "normal" majority; for them, research must focus on these "sick" individuals. This tug-of-war between the two approaches has thwarted major research support in either direction.

Reconciling Two Views

The dissension in the ranks of the alcoholism constituency has weakened its political and scientific influence; it behooves us to envisage ways through which the various philosophies of the alcoholism movement could be reconciled. One attempt at reconciliation could be achieved through the recognition that a public health approach could effectively be focused on the alcoholic population and perhaps some other subgroups which are particularly susceptible to alcohol, without thereby involving the entire society. The present paper will present some rationale for integrating the "disease concept" of alcoholism and the public health approach, leaving aside the thorny issue of consumption control. The concept of "disease control" to be presented in this paper may not only improve efforts at prevention and treatment, but it also may serve as a basis for a consensus amongst most alcoholism workers. It would thereby contribute to healing the rift between the two opposing forces in the alcoholism movement and may represent a rallying common goal for a funding rationale at the national level.

The host versus agent controversy in alcoholism illustrates a problem that has surfaced before in many medical fields. The example of tuberculosis is often used to highlight this issue. It has been pointed out that whereas in some societies most individuals are exposed to the Koch bacillus, very few develop disease forms of tuberculosis. It was concluded therefore that host factors are most important in this situation and the analogy was drawn with alcoholism (Gordon 1958). Obviously, most individuals in our society are exposed to alcohol but very few develop disease states in association with excessive drinking. Therefore, it has been argued the host is the most important factor determining whether or not an individual will become an alcoholic. This philosophy led to the emphasis on research concerning genetic and other factors that may influence the host response to alcohol. An entire new "alcoholism research" program developed by the Veterans Administration has in fact been instituted with an initial focus on "the etiology of alcoholism." One of its goals is to determine why certain individuals drink in excess and develop alcoholism whereas the majority of the population does not. The question is undoubtedly of fundamental importance but, if applied narrowly, such an approach would severely limit the scope of alcohol research. A popular research approach has been to try to define what makes an alcoholic different from a nonalcoholic either in behavioral, psychological or chemical terms. If the host concept were to be applied rigidly, such research would have to be limited to an impractical goal, namely the study of the alcoholic prior to his drinking, since any differences between the alcoholic and the nonalcoholic after the disease has evolved could be a consequence of the drinking rather than a preexisting situation. There would also be little justification in studying the effects of alcohol on the body except perhaps in alcoholics when evaluated prior to the development of alcoholism, an utmost difficult goal to achieve. Fortunately, the VA and various other alcohol research programs have been administered in a liberal way that has allowed for a somewhat wider approach but many investigators feel uncomfortable about having to support their broadly scoped research from funds derived from narrowly defined programs. The time has come to legitimize most of the studies carried out in the alcoholism field and to realize that as it is the case for most disease entities, the "host" versus the "agent" debate is a stale and somewhat artificial one and that both factors play an important role. The concept of "disease control" espoused here intends to reconcile the "host" and "agent" concepts by advocating classic public health approaches primarily focused on the heavily drinking population, avoiding thereby the politically divisive issue of consumption control applied to the general population.

That alcoholism has become a major public health problem can hardly be denied. Even after admission to an alcoholism treatment program, the mortality rate of alcoholics is two times greater than expected four years after initiation of therapy (Polich et al. 1980). If one focuses on one complication of alcoholism, namely cirrhosis of the liver (which in this country is primarily of alcoholic origin), one finds the astonishing statistic that in urban areas, this is now the third leading cause of all deaths in the active age group of 25 to 65 (Vital Statistics 1979). The impact of alcoholism on neonates and diseases of the brain, heart, gut, and endocrine system have been extensively illustrated in this volume. The disastrous effects of alcoholism on lethal road accidents is too well known to be emphasized here. Confronted with this major public health problem, our society could benefit from the consideration of successful techniques used in the past to combat other major public health problems. As previously mentioned, the example of tuberculosis has been used to justify the importance of the host in the development of this disease. The same example however could also be used to illustrate how a public health approach based on "disease control" could greatly benefit our efforts at prevention and treatment.

There are, in fact, many similarities between alcoholism and some other major public health problems which have confronted our society over the last century, such as tuberculosis. Of course, there are many dissimilarities between tuberculosis and alcoholism which will not be discussed here. The focus will be on some of the common aspects which might suggest possible avenues for intervention in the field of alcoholism. In both cases, the etiologic agent is known, the Koch bacillus for tuberculosis and ethanol for alcoholism. Both agents can affect most tissues of the body, but the most severe adverse effects usually occur in one organ, namely, the lungs for tuberculosis and the liver for alcohol. In both instances, exposure to the agent was or is widespread: a majority of the population had a positive reaction to tuberculin indicating contact with the Koch bacillus; similarly, the majority of the population consumes alcohol. However, only a minority of those exposed have complications.

Early Diagnosis

One of the successful approaches to the problem of tuberculosis has been the early detection of the affected subject through appropriate laboratory tests; alcoholism could lend itself to a similar approach. It is obvious that among the alcohol users there is a subpopulation of very heavy drinkers who are particularly at risk to the development of alcoholism and its complications. One goal should be the early detection of those people prior to their social or medical disintegration. This might be achieved through some chemical marker of heavy drinking, such as a blood test that could be performed by automated procedures on a large scale. There is reasonable hope that ongoing studies might be successful in the near future and may provide us with such a tool for the early detection of heavy drinking as discussed in this symposium. The availability of a marker for heavy drinking can be expected to be extremely useful in at least three situations:

1) to detect relapses in patients who have been rehabilitated;
2) to provide an objective means of assessing and comparing various treatment modalities;
3) for the screening of heavy drinking in populations at risk.

Another cornerstone of the public health approach to tuberculosis has been the early detection of its major complication through mass screening for pulmonary lesions. A similar approach may also be feasible in alcoholism through improved means to detect somatic changes at an early stage (i.e. liver lesions, hematologic changes, etc.). Unfortunately, commonly available tests are not very satisfactory. For instance, the severity of alcoholic liver injury is not reflected in standard liver-function tests; serum glutamic oxaloacetic transaminase and serum glutamic pyruvic transaminase levels are not strikingly elevated, even in alcoholic hepatitis, and the clinical and laboratory picture may thus mimic that of obstructive jaundice. Recently, however, better blood tests for the detection of alcoholic liver disease have been developed with improved sensitivity (for instance gamma glutamyl transpeptidase) or with enhanced accuracy which we found to be provided by glutamic dehydrogenase (Worner and Lieber 1980), as discussed elsewhere in this volume.

Defining Susceptibility

Because major complications such as cirrhosis do not develop in all heavy drinkers, one major task in an effort at prevention is to better define the susceptible population. We have approached this problem in our baboon model. As for the clinical situation, in only one-third of the baboons fed alcohol long-term has cirrhosis developed. We have now learned to recognize lesions in the liver which, already at a very early precirrhotic stage, enable us to predict which animals are prone to the rapid progression to the cirrhotic stage upon continuation of drinking. Indeed, in some baboons, perivenular fibrosis (fibrosis around the central veins) was observed as early as in the "simple" fatty liver stage, before more severe liver lesions developed. Moreover, sequential biopsies performed over a 6-year period showed that in all animals with perivenular fibrosis, septal fibrosis or cirrhosis developed after continued alcohol consumption. By contrast, neither fibrosis nor cirrhosis developed in animals without perivenular fibrosis within the same period of time (Van Waes and Lieber 1977). Thus, in this experimental model, the presence of perivenular fibrosis makes it possible already at the fatty liver stage to predict the subsequent evolution towards cirrhosis. If these results can be extrapolated to man, the presence of perivenular fibrosis should be regarded as a warning sign that permits identification of patients in whom cirrhosis is likely to occur. Obviously liver biopsies do not represent a practical tool for disease control on a large scale. It is not unreasonable to anticipate however that eventually blood tests might be developed that serve the same purpose of screening individuals who for some reason show a greater propensity to rapidly develop cirrhosis when exposed to the offending agent alcohol. Similarly, some of the central nervous system complications of alcoholism may occur preferentially in predisposed individuals, as illustrated by the preponderance of the Wernicke-Korsakoff syndrome in individuals with preexisting enzyme abnormalities (Blass and Gibson 1977). Detection of such or other preexisting abnormalities in high risk groups, i.e., heavy drinkers, may allow for selective intensive treatment efforts in such targeted groups, thereby enhancing the cost effectiveness of our therapeutic interventions.

Individual Susceptibility

Since alcoholism and its complications do not develop in all heavy drinkers, it is reasonable to speculate that some factors of heredity or individual susceptibility may play an important role. Ongoing studies on HLA factors illustrate such an approach. Obviously, research should be encouraged that may enable us to better understand these factors. Research efforts, however, should not be limited to such an approach because the question is an extremely difficult one, and we may not find an answer in the foreseeable future. For instance, we still do not know why in only a minority of subjects exposed to the Koch bacillus pulmonary cavities develop. We suspect that some genetic factor is important and that individual susceptibility plays a role, but despite a century of research these questions have not been elucidated. Yet, for all practical purposes, we have arrested the ravages of tuberculosis not only because of improved social and nutritional conditions, but also primarily by interfering with the etiologic agent, namely, the Koch bacillus, through the development of antimicrobials. Similarly, in the alcoholism field, although we may not understand why alcoholism develops in only some ethanol consumers and why liver disease develops in only a minority of heavy drinkers, we might nevertheless be able to intervene successfully if we could acquire a better understanding of how ethanol affects the chemistry of the body. It is not unreasonable to hope that better elucidation of the mechanism whereby alcohol affects various tissues might give us some handle with which to interfere effectively with the actions of ethanol, thereby perhaps altering the development of alcoholism and its major complications such as cirrhosis or brain damage. Currently, there are relatively few studies on the basic effects of alcohol. Actually, we sometimes hear that such fundamental studies might be somewhat irrelevant to alcoholism because they do not appear to deal directly with the problem of compulsive drinking and associated psychiatric pathology. Such a narrow viewpoint may be stifling. Indeed, in the long run, understanding of the biochemical effects of alcohol might actually provide us with a rational approach to the alcoholism problem by allowing us to intervene through interference with the action of ethanol. As for other fields, basic research (i.e., enhancement of our knowledge on the effects of alcohol on the body and the handling of ethanol by the body) may end up as the most productive research investment.

Differences Between Alcoholics and Nonalcoholics

Studies carried out over the last decade have shown significant biochemical differences between alcoholics and nonalcoholics. Further refinement of what makes an alcoholic different from a nonalcoholic not only in psychological but also in biochemical terms may be extremely useful as a basis for a biological approach to alcoholism. Eventually, extrapolation of these studies may provide us with some tools to detect those individuals prone to develop alcoholism prior to their exposure to alcohol. A possible example of such an evolution is provided by blood acetaldehyde. As discussed in this volume, for the same dose of ethanol, blood levels of acetaldehyde are higher in the alcoholic than the nonalcoholic (Korsten et al. 1975). More recently, extrapolation of this finding to relatives of nonalcoholics demonstrated a similar difference (Schuckit and Rayses 1979). The latter observation is still embroiled in methodological controversies; nevertheless, it is of interest since it illustrates how studies of consequences of alcohol abuse may eventually lead to a better insight in detecting individuals prone to develop alcoholism and what determines such behavior.

Of course, alcoholism is multifaceted. Behavioral, social and psychologic difficulties play a major role and our focus on recognition and correction of these problems should continue. However, we cannot escape the fact that conventional approaches to alcoholism based on these traditional concepts have not sufficed to stem the tide of alcoholism. On the other hand, attempts at elimination of the etiologic agent (through prohibition) have failed. One virtue of the biologic outlook is to make possible the limited public health strategy based on disease control as outlined before (Lieber 1978) and further defined here.

Of course, it would be naive to anticipate that all aspects of alcoholism could be solved by a single approach. But if greater reliance on public health strategy were to result in only moderate reduction in the incidence of alcoholism, or even if it were only to diminish some of its severe complications (i.e., cirrhosis or brain damage), such a partial success could be expected to have a significant impact on productivity, morbidity, and mortality. Public health intervention, in conjunction with the more traditional treatment and grass root movements (such as Alcoholics Anonymous) may enable us to get a better handle on the problem of alcoholism.

The last three decades have seen significant advances in our understanding of the medical complications of alcoholism. Major advances resulted from our recognition of the toxicity of alcohol in various tissues particularly the liver (Lieber 1980) and some of the mechanisms involved. The knowledge and experimental tools acquired, particularly the experimental models of chronic alcohol consumption, allow us now to contemplate a truly public health strategy in the alcoholic. Classically, major efforts have been directed at establishing the disease concept of alcoholism and in determining what makes people drink excessively or predisposes them to this loss of control. It is apparent that an answer to that question might enable us to develop a handle on the problem of alcoholism itself and that therefore, this approach should be continued with the highest priority. Such a difficult question, however, may not be resolved in the immediate future. It is of utmost importance that the lack of resolution of this basic question does not deter us from other approaches to the problems of alcoholism (such as disease control advocated here) which may alleviate much of the individual suffering and the social consequences.

Summary

In summary it is being advocated that a disease control strategy toward alcoholism and its medical complications be added to our main approach based on treatment of underlying psychological and behavioral problems. This public health approach is based on early detection of alcoholism (utilizing, in part, biochemical markers of heavy drinking); screening, among heavy consumers, for signs of medical complications (through the use of improved blood tests); and reducing the task of treatment to a manageable size by focusing major therapeutic efforts on susceptible subgroups, for instance those recognized to be prone to the development of major sequelae (i.e., cirrhosis of the liver or brain damage) upon continued heavy drinking.

The benefits of this "disease control" approach are multiple. It offers research goals which are realistic and achievable in the foreseeable future and which, though they may not solve the fundamental problem of alcoholism, would nevertheless clearly alleviate the suffering of the alcoholic and the public health impact of alcoholism on our society. This approach would also legitimize much of the research which is currently carried out in this field and is supported by public agencies such as the National Institute on Alcohol Abuse and Alcoholism. A major advantage of the disease control concept is a potential for reconciliation between classic advocates of public health approaches who wish to focus on the agent, namely alcohol and those who feel strongly that all efforts should be directed towards the susceptible host, namely the alcoholic. The antagonism between the two groups has been exacerbated, in part, by efforts on behalf of public health advocates to control alcohol consumption through measures that involve the entire population. The approach proposed here, namely the application of public health measures primarily to the alcoholic population itself may help reintegrate public health advocates into the alcoholism movement without evolving fears of "neo-prohibitionism." An "ecumenical" approach may provide alcoholism research with the backing needed to achieve status, stature, and support equal to that enjoyed by other public health problems in this country.

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